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Causes and consequences of ejaculate size in *Callosobruchus maculatus* beetles

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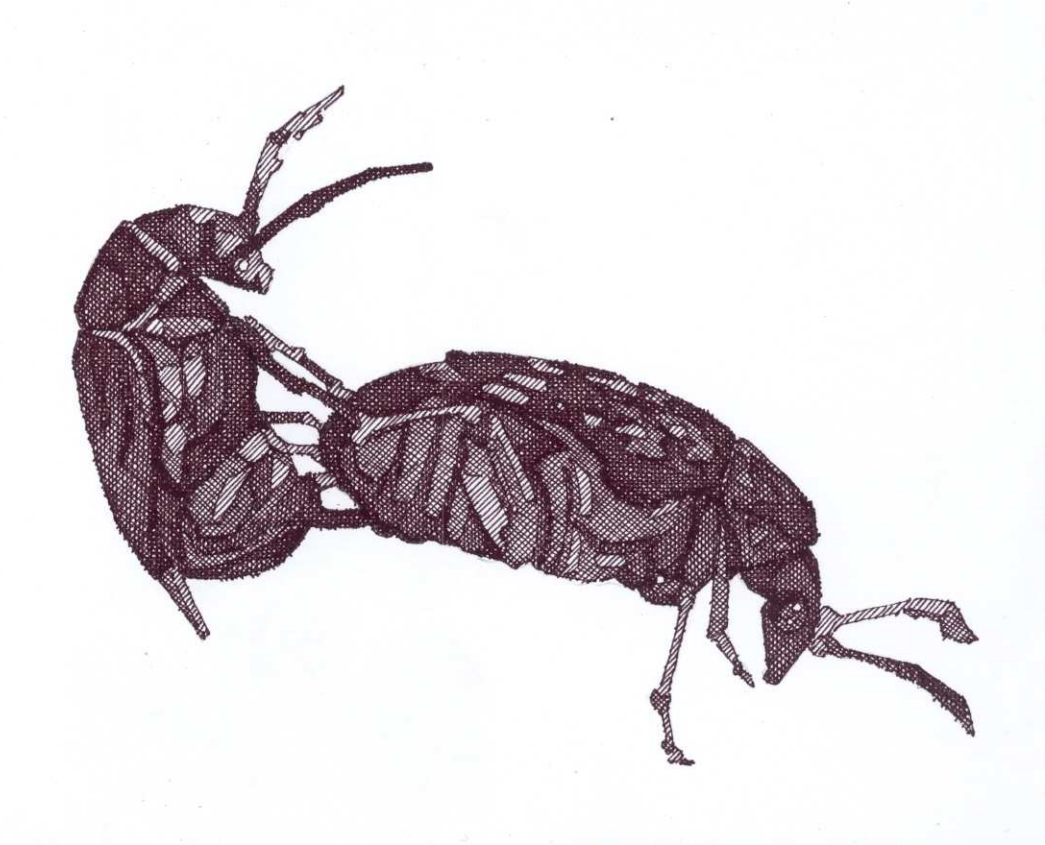

A thesis submitted for the degree of

Doctor of Philosophy

in the

Institute of Evolutionary Biology

University of Edinburgh

2012

Declaration

This thesis has been written entirely by me, Fiona Lethbridge. All the work for this thesis, including experimental design, data collection and analysis, was done by me, with the exception of:

Chapter 6, which was carried out in collaboration with an honours student, Ian Skicko. I designed the experiment and provided tuition in the laboratory, and stipulated how data should be collected. Ian Skicko collected the data under my supervision. I analysed the data myself and the results and conclusions I present in this thesis are those I have drawn myself.

I confirm that I have not submitted any of the work in this thesis for any other degree or professional qualification.

Signed

Fiona Lethbridge

Date 14 June 2012

Acknowledgements

Many thanks to Professor Matthew Gage and Doctor Penny Haddrill for reading this thesis, and for examining me in a thorough, engaging and enjoyable way during my viva.

Thanks to Mum and Dad for letting me live in their house and eat their porridge while I finished writing my thesis (and for support, encouragement and dementia throughout). Thanks indeed to all the Lethbridges; Zoe and Martin, Naomi, and Rory, for providing places to escape to during these past few years. Enormous thanks to Chris Wood.

Pete has often provided invaluable help in the lab; thanks to him, and also to Vernon for the use of his equipment. Thanks to Ram Vasudev and Paul Eady for sperm count tuition, and to honours student Ian Skicko, for contributing to Chapter 6. Huge thanks are due to the ever-helpful and lovely ladies in the Ashworth office, especially Carole and Claire. Thanks immensely to Josephine Pemberton, Brian Charlesworth, and whoever else made the excellent decision that I should be given some money in order to attend a conference in Australia (go snorkelling with Manta rays). Thanks to Naomi Lethbridge (www.naomilethbridge.co.uk) for producing the illustration for the cover page.

Ashworth has been a great place to be, largely because of the people in it. Before they deserted me, I had wonderful times with Olivia, Laura K, Anjie and Adel; they continue to support me from various locations on this globe, and I miss them a lot. A special thanks to Olivia, who illustrated her faith in my ability to finish the PhD by giving me a 'viva present' over a year ago! I look forward to opening it. I also miss Alex, who helped me settle in as a Colegrave apprentice. My postgraduate cohort have been supportive and drunken throughout; thanks to Rebekah, Harriet, Matty (and his filth), Johanna, Lynne, Marisa and Paul. Indeed all inhabitants of Ashworth have been lovely; thanks to everyone. I am appreciative of wonderful office-mates, past and present, including Chrissy, Hanna, Marisa, Heidi, Stu, Pedro, Jen and Jay. A special and rather sizeable thanks also go to Penny, Dan and Hamish, for letting me swear about things in the Earl of Marchmont.

To flatmate Laura, thanks for providing a refuge away from science, and to Lauren, thanks for the VLEMWy. To many other Edinburgh-based friends; Christine, Tallah, Ianthe and Anna; I am grateful for your excellence. Thanks to Paul for providing rock-filled hours on Fresh Air. I owe a large portion of thanks to the Bright Club Edinburgh lot - Dr DRE, Charlotte, Sarah, Andy, Robin, Steve, Dan, Zara and Susan, for allowing me, and indeed, rather alarmingly, encouraging me to take to the stage to sing about sperm competition in insects and make jokes about damaging genital morphology. Thanks ever so much to the BioPOD team - the excellent Cheryl, James and Eugene, and the supportive Dan Nussey, for reminding me that science can (occasionally) be fun.

I'd very much like to thank the British taxpayer for contributing to scientific research funding, and to BBSRC for 3.5 years of studentship.

Finally, a really quite gigantic quantity of thanks go to my marvellous supervisors, Nick Colegrave and Emma Cunningham (the Dream Team). They have both encouraged me enormously, and I certainly wouldn't have finished this (the PhD, not the acknowledgements) without them. Nick has provided support, amusement, wigs and friendship over the last few years; I would like him to know how much I appreciate that. I am also very grateful to Becky, and to Colegraves of all sizes, for often welcoming me into their home (and their Wendy house), and for feeding me between games of hide and seek. Nick read thesis chapter drafts in evenings and during weekends, which I know he didn't have to do; thanks Nick. I only cried at Nick once (ed - twice; happened again since writing this), and he handled it impeccably (offered me vodka); I am extremely grateful for his unfailingly reliable support and understanding. Nick, if I could afford an iPad, I'd give you one to add to your collection, as a thanks for all you've done for me. Unfortunately, because of the recession, you'll have to make do with my gratitude.

Abstract

Post-copulatory sexual selection is a strong evolutionary force, affecting morphological and behavioural traits in males and females in species with polyandrous mating systems. Many insects are subject to sperm competition; sperm from rival males compete to fertilise ova. Since sperm are finite, males should allocate them economically, tailoring ejaculate allocation to suit the reproductive potential of individual matings. Theory suggests when sperm competition risk is high, males should increase sperm numbers to achieve greater reproductive success than their rivals, but evidence of this expected fitness consequence of ejaculate allocation is largely lacking. In this thesis, I use *Callosobruchus maculatus* beetles to investigate the causes of ejaculate allocation patterns, and to examine whether ejaculate allocation does affect male reproductive success. In Chapter 3, I investigate the effect of rival male presence on ejaculate size and find that, while males grouped with rivals as adults produce bigger ejaculates, their increased effort unexpectedly does not lead to increased reproductive success. In Chapter 4, I examine whether larval conditions also affect ejaculate size, and find that, contrary to sperm competition theory, males reared under dense conditions produce smaller ejaculates than those reared solitarily, and that male reproductive success is consequently elevated in males reared at low larval densities compared to those reared at high densities. In Chapter 5, I then demonstrate that ejaculates produced by low density males contain more sperm than ejaculates produced by high density males, suggesting males do not respond to sperm competition level represented by larval density, but instead suffer resource limitation when reared at high density. In Chapter 6, I investigate the effects of water provision on ejaculate size, and find that males given water produce larger ejaculates, and females given water receive smaller ejaculates. Finally, I link my findings with those of other studies, and suggest my most important result is that plasticity of ejaculate allocation cannot be assumed to be an adaptive behaviour; studies directly measuring the fitness effects of male ejaculate allocation are needed, even when observed patterns conform to theory.

Contents

Chapter 1. Introduction

1.1. Sexual selection

Sexual selection is an important evolutionary force, driving changes in behavioural, morphological and physiological characteristics of males and females. Competition between individuals for mates (intrasexual selection), and mating preferences of the choosy sex (intersexual selection), can select for changes in male and female traits over evolutionary time within species. In species with high levels of male-male competition for access to females, sexual selection can act on a variety of traits including male body size (Parker 1992), weaponry (Tomkins and Simmons 2000), ornamentation (Andersson 1986) and testes size (Gomendio *et al* 1998; Gage 1994; Hosken 1997; Stockley *et al* 1997; Hosken and Stockley 2004). For example, in species in which there is a high degree of polyandry, males are often selected to grow larger testes than males in closely related monogamous species (Gomendio *et al* 1998; Gage 1994; Hosken 1997; Stockley *et al* 1997; Hosken and Stockley 2004). Males with characteristics that make them most successful in achieving fertilisations achieve greatest reproductive success; their offspring therefore inherit genes enabling them to compete more successfully against rival males, and granting them the traits favoured by females, therefore they too will achieve greater reproductive success than other males in the population.

Selection will often act differently on males and females - males generally achieve greatest reproductive success by maximising the number of females with which they mate, while females are constrained by offspring production (Bateman 1948), and so do not always benefit from mating with multiple males. Anisogamy has led to the difference in the way selection acts on males and females - ova are large and few, relative to numerous and tiny sperm (Parker *et al* 1972), so males are generally selected to maximise numerical

productivity of offspring using their numerous gametes, whereas females are generally selected to maximise offspring fitness by providing resources via large ova.

Many of the manifestations of sexual selection are visible and obvious at the pre-mating stage; bigger males generally achieve more matings (Pilastro *et al* 2002), males with bigger horns might achieve more matings (Tomkins and Simmons 2000), and males with more desirable ornamentation or appearance often achieve more matings (Brooks and Couldridge 1999). However, sexual selection does not stop once mating has been achieved. The relative reproductive success of males continues to be affected by processes occurring after copulation, as multiple males mating with the same female continue to compete for fertilisations; this is post-copulatory sexual selection.

1.2. Post-copulatory sexual selection

Post-copulatory sexual selection occurs in species in which females mate multiply with different males, and produce broods of offspring that can potentially be fathered by any male that a female copulates with (Parker 1970). Such selection can have incredibly important effects on a wide range of physiological and behavioural traits in both males and females. The reason that multiple mating will often be selected for in males is clear, but the selective advantages of multiple mating in females are less obvious; while in males maximising mate number increases reproductive success, in females this is not always the case (Simmons 2001). The most obvious reason for females to mate multiply is to obtain sufficient sperm to fertilise all available eggs. In insects, females can become sperm-limited; in some cases evidence has shown female fecundity increases with multiple mating (Arnqvist and Nilsson 2000). Polyandry could therefore protect against infertility due to the insemination by some males of inviable sperm, or degradation of sperm when in storage for long periods of time. In many insects, however, females store sperm within their reproductive tracts and, in some cases, a single insemination provides enough sperm to fertilise their lifetime supply of eggs

(Eady 1995). For such females, another possible reason for multiple mating might be to increase the likelihood of mating with a male with more favourable genes that those of previous mates, which might benefit females indirectly through increased offspring fitness (Simmons 2001). Another reason for multiple mating in females might be to overcome potential genetic incompatibility. For example, in the field cricket, *Gryllus bimaculatus*, females that mate multiply with different males achieve greater offspring hatching success than females that mate multiply with the same male, but there are no patterns among males in the hatching success of their mates' eggs (Tregenza and Wedell 1998), suggesting females individually benefit from polyandry due to the increased likelihood of mating with a male with genes that are more compatible with their own.

Ejaculates contain components that allow fertilisation of eggs (the sperm), as well as other substances aiding motility of gametes and their safe delivery into the female reproductive tract, and other constituents that can affect the chance of achieving fertilisation. In principle, sexual selection can act on any, or all, of these components, depending on the species and its mechanism of post-copulatory sexual selection.

1.2.1. Sperm competition

The most obvious ejaculatory component affected by sexual selection is the quantity or morphology of sperm. An important aspect of post-copulatory sexual selection in many species is sperm competition; sperm from more than one male compete within the female reproductive tract for fertilisation of the same set of ova (Parker 1970) (or, in externally fertilising species, compete in the environment, for example in externally-spawning fish) (Parker 1970), leading to differing reproductive success among males of a population, depending on the resources they invest. Sperm competition can potentially occur in any species in which females store sperm from multiple males at the same time. In many insects, female sperm storage organs are called spermathecae, which vary widely in morphology

between species. Some spermathecae have fixed capacity, and others can stretch (Eberhard 1996); most will accommodate multiple ejaculates (Simmons 1986), although the composition of the ejaculate mixture will depend on numerous factors including the order in which they are inseminated, the morphology of female storage, and the way in which ejaculates mix after insemination.

In species where sperm competition occurs, selection should act on males to both avoid sperm competition, by adopting traits likely to prevent or postpone females mating subsequently with rivals, and to be more effective when they do encounter sperm competition, by adopting traits that will increase how many eggs they can fertilise relative to the ejaculate of a rival male. Such traits affected by this post-copulatory sexual selection could include male behaviours directed towards females, physical attributes of male genitals, and physiological traits of the ejaculates males inseminate. Characters which appear to have evolved to avoid sperm competition occur in a diversity of organisms, and include post-copulatory mate-guarding (Carroll 1991), the production of mating plugs to prevent future inseminations (Simmons 2001), the possession of genital structures to remove previously-inseminated ejaculates (Orr 1995), and the insemination of chemical substances affecting female oviposition and re-mating behaviour (Simmons 2001). Although these adaptations can confer at least partial avoidance of sperm competition, they still affect the outcome of post-copulatory sexual selection. Of course, if selection gives rise to male adaptations that completely avoid sperm competition, such as some copulatory plugs, then ejaculates themselves might not be selected to have competitive traits.

When multiple mating does occur, sperm from multiple males can compete in different ways within the female reproductive tract, depending on factors including female physiology, species ejaculate characteristics, and the action of sperm once within females (Simmons 2001). In some species, sperm from different males mix completely after insemination and compete simply in a raffle process; the outcome of competition depends only on the relative

numbers of sperm inseminated by different males (Parker *et al* 1990). In other cases, competition occurs as a 'loaded raffle' (Parker 1990); as well as relative numbers of sperm being important, the outcome of competition can also depend on the positioning of ejaculates within the female reproductive tract, the order in which different males mate, or the different competitive abilities of sperm from different males. In species in which the outcome of sperm competition can depend on the order in which ejaculates are inseminated, advantage is gained by being in the favoured position. This is known as sperm precedence (see Simmons 2001 for examples); in some species first-mating males have precedence (Harano *et al* 2008) and in others last-mating males have precedence (Lewis and Jutkiewicz 1998; Kock and Sauer 2007). If spermathecal morphology means different ejaculates do not overlap much, immediate direct competition between sperm from rival males might be avoided, but over time competition for fertilisation of eggs can still occur, so this is still post-copulatory sexual selection. One mechanism leading to sperm precedence is sperm stratification - ejaculates are positioned in layers within the female spermatheca, and one ejaculate gains fertilisation precedence due to its position; often the last ejaculate to be inseminated is the first to be used for fertilisation (Simmons 2001). In some species, males have the ability to physically displace sperm previously inseminated by rival males, either by removing it entirely from the female (Parker and Simmons 1991) or by moving it to a position where it is less likely to successfully fertilise ova. Sperm removal has been demonstrated in insects that use specialised genital morphology to dislodge or pull out rival sperm (Sherman 1983). Sperm displacement has also been found to occur in some species that flush out a previously-inseminated ejaculate using the bulk of their own insemination (Rondeau and Sainte-Marie 2001). Males of some insects have the ability to incapacitate sperm from rival males using chemical substances within the ejaculate, or morphological adaptations of the sperm themselves; this gives them fertilisation precedence over their rival males. In *Drosophila melanogaster*, for example, seminal fluid products have been shown to render ineffective sperm inseminated by previously-mating rival males (Harshman and Prout 1994).

Sperm competition level is divisible into two components; sperm competition risk and sperm competition intensity (Parker 1970). Sperm competition risk is the chance that the ejaculate of a mating male will have to compete with a rival ejaculate; two ejaculates will be engaged in competition. Sperm competition intensity is the number of rival ejaculates that might compete for fertilisations in species that generally have high levels of female re-mating (Parker 1970). The adaptive responses of males to these levels of sperm competition can be different; males experiencing increased risk of sperm competition should increase their reproductive effort for that mating, in order to increase their chances of achieving fertilisation when in competition with a rival (Parker *et al* 1997). When the intensity of sperm competition varies, males should give their greatest resource allocation when one competitor is present, but allocation should decrease when the number of competitors is two or larger. This is due to the diminishing returns associated with investing more in a situation that is likely to have an unfavourable outcome (Parker *et al* 1996); males should instead conserve reproductive resources for a potential future mating, in which the intensity of sperm competition is lower. The way in which sperm competition affects the way males allocate sperm to their ejaculates therefore depends on the level of polyandry within the species, and on whether the risk or intensity of sperm competition varies over the lifetimes of males.

There is also evidence that sperm morphology can be affected by post-copulatory sexual selection; in the cricket, *Gryllus bimaculatus*, sperm size is variable. Males producing smaller, but more numerous sperm achieve greater reproductive success (Gage and Morrow 2003). This suggests that, in this species, sperm number is more important than sperm size, so sperm size is sacrificed in order to maintain numerical superiority.

1.2.2. Seminal fluid

Some seminal fluid substances can induce a refractory period when inseminated in females, preventing them from re-mating again for a time after the initial copulation. In species whose

ejaculates contain such components, males should be selected to maximise the quantity of these substances in their ejaculates, because increasing the period of female non-receptivity will increase the length of time for which sperm competition can be avoided. In addition, increasing the period of non-receptivity is likely to increase the number of eggs females lay before re-mating; therefore maximising the proportion of a female's lifetime supply of eggs that are fertilised by the focal male alone. Seminal products can also stimulate oviposition and even increase the numbers of eggs females lay. In some crickets, for example, within their ejaculates, males transfer the enzyme prostaglandin synthetase along with arachidonic acid, which, in females, is converted to prostaglandin in the spermatheca, and is taken up by ovaries (Simmons 2001). This prostaglandin stimulates females to oviposit (Simmons 2001); males might therefore be selected to increase the quantities of these substances in their ejaculates, in order to maximally stimulate females to lay eggs fertilised by them.

In some moths, juvenile hormone affects female oviposition behaviour, stimulating egg maturation and oviposition (Simmons 2001), for example, in the tobacco budworm, *Heliothis virescens* (Park *et al* 1998). Males inseminate females with juvenile hormone via their ejaculates (Simmons 2001); therefore males should be selected to maximise its quantity in their ejaculates to increase the numbers of eggs females lay when only their sperm is being used for fertilisation. Also in some moths, substances in male ejaculates antagonise a pheromone released by females to attract mates, so that after mating females have a smaller chance of attracting a second mate (Simmons 2001), such as in the corn earworm, *H. zea* (Raina 1989). Males might therefore be selected to increase allocation of this substance, pheromonostatic factor (Simmons 2001), in their ejaculates, so that female re-mating is delayed, and males are therefore less likely to face sperm competition.

In some butterflies, female receptivity to re-mating is inhibited by the activation of stretch receptors in the reproductive tract, and it has been demonstrated in some cases that insemination of larger spermatophores prolongs the period of non-receptivity, such as in the

small white butterfly, *Pieris napi* (Kaitala and Wiklund 1994); males might therefore be selected to inseminate larger ejaculates, in order to avoid sperm competition from inseminations from subsequent males.

In *Drosophila melanogaster*, a large number of proteins produced by male accessory glands are included in ejaculates, some of which affect female behaviour (Wolfner 1997). One protein, esterase 6, is transferred into females during copulation, and decreases female receptivity to re-mating (Richmond *et al* 1980). Another substance contained in *D. melanogaster* ejaculates that affects female re-mating and oviposition behaviour is sex peptide (Chen *et al* 1988), and other accessory gland proteins have been suggested to have the ability to kill or incapacitate previously-inseminated sperm (Rice 1996). *D. melanogaster* males also include a pheromone in their ejaculates that reduces female attractiveness to future potential rival male mates, and is therefore an antiaphrodisiac (Tomkins and Hall 1981). Post-copulatory sexual selection will likely select male *D. melanogaster* to increase the allocation of these various substances in their ejaculates, in order to avoid, and more effectively engage in, sperm competition.

As well as inseminating chemicals in their ejaculates that affect female behaviour, some males can provide nutrition to females during mating, in the form of nuptial gifts. By including in their ejaculates nutrients or water, males can increase the longevity or fecundity of their female mates, and can also contribute to the fitness of the offspring they produce. In species in which post-copulatory sexual selection acts, providing nuptial gifts might be adaptive for males because a well-provisioned female might lay more eggs, or lay eggs at a faster rate following insemination, or might not need to re-mate subsequently with rivals to obtain more resources. By providing nuptial gifts, males might therefore achieve greater reproductive success. The effects of nuptial gift provision have been demonstrated in a number of insects, including many species of cricket. During mating, male bushcrickets transfer, along with their spermatophore, a nutritious spermatophylax, which is eaten by

females (Simmons and Kvarnemo 1997), and can therefore influence female fitness and, consequently, the fitness of their offspring.

1.2.3. Mating plugs

In some insects, during copulation males inseminate substances that form physical barriers within the female reproductive tract, to prevent or delay further inseminations; these mating plugs are most common among butterflies (Simmons 2001). Because mating plugs reduce the likelihood of females re-mating with rivals, their production is selected by post-copulatory sexual selection to avoid sperm competition. Mating plugs remain within the female reproductive tract only temporarily; in order for females to be able to lay eggs, the plugs have to be removed. It is therefore not necessarily adaptive for males to produce mating plugs that persist for longer within females, because if they did they would be damaging their own reproductive success. In some species, females can re-mate within a short time window after the mating plug has been deposited, but before it has hardened (Simmons 2001). Males might therefore be selected to increase the speed at which their plugs harden.

Mating plugs vary considerably in morphology between species; in most butterflies, mating plugs are extensions of the spermatophores that males transfer to females during copulation (Simmons 2001). Because all males within a species are under the same selection pressure to produce mating plugs, in some cases adaptations might arise that allow males to remove mating plugs inseminated by their rivals (Parker 1984). Evidence of such adaptation has been seen in some butterflies (Orr 1995). In addition, in many insects, sperm are passively lost from the female reproductive tract after mating (Eberhard 1996); sexual selection might therefore also act on males to produce effective mating plugs, so that fewer of their own sperm are lost, and so the reproductive potential of their ejaculates are maximised.

1.2.4. Testes size

In species and populations that are subject to strong post-copulatory sexual selection, males can be selected to grow large testes. Testes produce sperm, so having larger testes is adaptive because it can increase the numbers of sperm males can produce and inseminate, thus enabling them to engage more effectively in sperm competition. There are examples of this in nature; closely related species with different mating systems can vary in testes size, due to different selection pressures. Males of the polyandrous vole, *Microtus ochrogaster*, grow larger testes relative to their body sizes than the closely related monandrous vole, *M. pinetorum* (Gomendio *et al* 1998), due to the stronger post-copulatory sexual selective pressure acting on males in the species in which females commonly re-mate with rivals.

1.3. Ejaculate adjustment

It is clear that selection can act on traits such as testes size and ejaculate contents in response to the general level of post-copulatory sexual selection that occurs in a species. However, in many species, the degree of sexual selection might vary dramatically, either from generation to generation within a population, or even from mating to mating within an individual male's lifetime. Males that can potentially detect such variation, and adjust their ejaculates accordingly, would be expected to be favoured by selection.

It could be beneficial to males to alter various components of their ejaculates in response to varying levels of post-copulatory sexual selection, in attempt to maximise their reproductive success. There is evidence of such ejaculatory allocations in many species of insect, as well as in some birds, fishes and mammals. Which component of the ejaculate is altered can depend on how success under post-copulatory sexual selection is best achieved in that species.

1.3.1. Altering sperm numbers

It used to be commonly assumed that sperm were cheap to produce, and limitless. However, more recent evidence suggests sperm are in fact finite (Nakatsuru and Kramer 1982), and the cost of their production is not insignificant (Dewsbury 1982). When placed under nutritional stress, male Indian meal moths produce fewer sperm than when provided with adequate nutrition (Gage and Cook 1994), confirming the non-trivial cost of sperm production. Because of their limited nature, it is not always adaptive to maximise the number of sperm in an ejaculate; if there is little chance of achieving significant reproductive success, it can be more economical to allocate few sperm and instead save more for future, more profitable matings. Indeed there is evidence in many species that males do adjust the number of sperm they allocate to matings depending on a variety of factors.

Male rats, *Rattus norvegicus*, alter the number of sperm they allocate to ejaculates depending on their socio-sexual surroundings (Pound and Gage 2004); when a rival male is present, males inseminate more sperm than when rivals are absent (Pound and Gage 2004). Similarly, in the sand martin, *Riparia riparia*, sperm numbers are greater when rival males are present than when they are absent (Nicholls *et al* 2001). Male grass gobies (*Zosterisessor ophiocephalus*) and black gobies (*Gobius niger*) exist as two morphs; sneakers and guarders (Pilastro *et al* 2002). Sneaker males always face sperm competition, because the females they sneak mates with will also mate with guarding males. When sneakers mate in the presence of one rival, they increase the numbers of sperm they allocate, and when the number of rivals present increases beyond one, they decrease their sperm allocation (Pilastro *et al* 2002), due to the diminishing reproductive returns of allocating sperm when sperm competition intensity is high.

In the fowl, *Gallus gallus*, males allocate sperm numbers differentially, depending on both male role and rival presence. Dominant males are sometimes able to prevent females mating

with rivals, whereas subdominant males are not; subdominant males therefore face a greater level of sperm competition (Pizzari *et al* 2003). Dominant males inseminate the largest numbers of sperm when mating in the presence of three males, because rival presence represents a greater risk of sperm competition (Pizzari *et al* 2003). Conversely, subdominant males allocate most sperm when mating in the presence of only one rival, and decrease sperm numbers as rival number increases beyond one; for subdominant males, a large number of rivals represents intense sperm competition, so sperm is conserved for future, less intense matings (Pizzari *et al* 2003).

Many species of cricket also alter sperm numbers depending on socio-sexual surroundings (Gage and Barnard 1996; Schaus and Sakaluk 2001). In the house cricket (*Acheta domesticus*) and the decorated field cricket (*Gryllodes supplicans*), males increase sperm number allocation when rival numbers increase (Gage and Barnard 1996). In the spring field cricket (*Gryllus veletis*), males allocate the largest numbers of sperm when mating in the presence of one rival, and decrease sperm numbers as rival numbers increase (Schaus and Sakaluk 2001). These varying sperm allocation patterns in different crickets might be due to species-specific mechanisms of post-copulatory sexual selection. In the Mediterranean fruit fly, *Ceratitis capitata*, males mating in the presence of a rival inseminate more sperm than those mating in isolation (Gage 1991); in this case the presence of one rival indicates an increased risk of sperm competition, so males react by increasing their sperm allocation.

As well as rival male presence, other factors can influence sperm allocation tactics. In the adzuki bean beetle, *Callosobruchus chinensis*, males from polyandrous strains allocate more sperm to matings if they develop alongside other larvae than if they develop alone (Yamane and Miyatake 2005; 2008). The presence of other conspecifics during larval development could be an indicator of population density or sperm competition level.

In the stickleback, *Gasterosteus aculeatus*, males alter their sperm allocation depending on the behaviour of surrounding rival males (Zbinden *et al* 2003). When surrounding males exhibit courtship behaviour, males increase the number of sperm above the number allocated when surrounding males instead exhibit brooding behaviour (Zbinden *et al* 2003).

Sex ratio can also cause males to alter their sperm allocation to matings (Dewsbury 1982; Rondeau and Sainte-Marie 2001; Evans *et al* 2003). In the snow crab, *Chionoecetes opilio*, males increase sperm numbers when the sex-ratio is male-biased (Rondeau and Sainte-Marie 2001). In the eastern mosquitofish, *Gambusia holbrooki*, males also allocate more sperm when there are more males than females (Evans *et al* 2003). Sex ratio might indicate likely sperm competition risk; if there are more males relative to females, the risk of having to compete for matings and fertilisations is increased.

Female mating status, too, can cause males to alter their sperm allocation. In the moth, *Plodia interpunctella*, males can detect female mating status, and allocate more sperm to females that have previously received a large ejaculate from a rival (Cook and Gage 1995), in attempt to more effectively engage in sperm competition. In the blue crab, *Callinectes sapidus*, males allocate more sperm when mating with a previously-mated female than when mating with a virgin (Jivoff 1997), and in the snow crab, *C. opilio*, males increase sperm number as the number of a female's previous mates increases (Rondeau and Sainte-Marie 2001) . Conversely, males of the swallowtail butterfly, *Papilio machaon*, allocate more sperm when mating early in the breeding season (when females are likely to be virgins) than later in the season (when females are likely to be previously-mated) (Svärd and Wiklund 1986). Different mechanisms of post-copulatory sexual selection in these different species are likely to account for the differences in sperm allocation patterns - in some cases, the greater potential reproductive returns offered by virgin females makes it adaptive to invest more heavily in them (as is the case in the swallowtail butterfly), whereas in others, the greater degree of sperm competition in previously-mated females means it is adaptive to

invest more heavily in them (as is the case in the blue crab and the snow crab), in attempt to more effectively engage in competition with rival sperm. In addition, in the snow crab, larger ejaculates more effectively displace previously-inseminated sperm (Rondeau and Sainte-Marie 2001); it is therefore adaptive for males to increase allocation when mating with a non-virgin female, in attempt to achieve greater fertilisation precedence, rather than allocate more sperm to a virgin, because a subsequently-mating male will likely achieve greater precedence.

Some males also tailor sperm numbers to individual females differently at different matings. Males of the fowl *Gallus gallus* reduce the numbers of sperm they allocate the more times they mate with a particular female, eventually refusing to mate with her at all (Pizzari *et al* 2002; Pizzari *et al* 2003). However, this is not just due to males suffering sperm depletion, because, when mating with a novel female, males increase their sperm allocation (Pizzari *et al* 2002; Pizzari *et al* 2003). This is known as the Coolidge effect (Dewsbury 1981). Similarly, Adélie penguin (*Pygoscelis adeliae*) males decrease sperm numbers when mating with their social partner, and allocate more sperm to extra-pair copulations (Hunter *et al* 2000). In both species, novel females might represent an increased risk of sperm competition, while paternity assurance with a permanent social partner or familiar female might be greater, so fewer sperm are required. Alternatively, it might be that males are assured of future copulation opportunities with their social partners, so have no need to inseminate larger numbers of sperm than the minimum required to fertilise all available eggs at that time, whereas future copulations with extra-pair females are unlikely, so males inseminate as many sperm as possible during the one mating opportunity they are likely to get.

Some males also allocate sperm differently depending on other attributes of females they mate with. Males of the bluehead wrasse, *Thalassoma bifasciatum*, increase sperm numbers as female body size increases (Rasotto and Shapiro 1998), as do male bucktooth parrotfish, *Sparisoma radians* (Shapiro *et al* 1994; Marconato and Shapiro 1996). Similarly in the moth

P. interpunctella, males detect female body size by assessing abdominal size, and allocate more sperm to larger females (Gage 1998), which makes sense in terms of life history theory, since larger females are often more fecund. Conversely, male bushcrickets (*Kawanaphila nartee*) allocate fewer sperm to larger females (Simmons and Kvarnemo 1997). Again, in different species, the adaptive pattern of sperm allocation can be different, possibly depending on whether sperm competition risk or intensity is the predominant force. In the bushcricket, for example, because courtship is role-reversed (females compete for matings with males), larger females achieve more matings with more rivals (Simmons and Kvarnemo 1997), therefore sperm competition is likely to be intense, so it is adaptive for males to reduce their sperm allocation to these large females.

Female age, too, can affect male sperm allocation strategies. In the dung fly, *Sepsis cynipsea*, males transfer more sperm to older females than to young females (Martin and Hosken 2002), possibly due to the greater risk of sperm competition represented by older females, which are more likely to have mated previously with rivals.

1.3.2. Altering sperm type

In some species, males produce sperm of different types. Depending on the function of these different sperm, it might be adaptive to allocate them differently, depending on the level of post-copulatory sexual selection. Many butterflies and moths produce both eupyrene (fertilising) and apyrene (non-fertilising) sperm (Cook and Gage 1995; Cook and Wedell 1999). Although the function of apyrene sperm is still under debate, it is thought they might be involved in aiding motility of eupyrene sperm within the female reproductive tract (Katsuno 1977), or they might delay female re-mating (Cook and Wedell 1999). When mating with young females, male *Plodia interpunctella* meal moths inseminate greater numbers of both eupyrene and apyrene sperm than when mating with older females (Cook and Gage 1995). By reducing eupyrene numbers allocated to older females, males save

fertilising potential for matings with younger, more fecund females, and by inseminating more apyrene sperm into younger females, the spermatheca can be more effectively filled, and consequently re-mating can be delayed (Cook and Gage 1995). Armyworm beetles, *Pseudaletia separata*, also produce both eupyrene and apyrene sperm, and can allocate them differently depending on their circumstances. Males reared in groups as larvae produce greater numbers of apyrene sperm than those reared solitarily, but eupyrene sperm numbers do not differ (He and Miyata 1997). It is suggested this is an adaptive response to the greater risk of sperm competition represented by greater larval density - as apyrene sperm might be cheaper to produce (Silbergleid *et al* 1984), by increasing their numbers, males can more effectively engage in sperm competition if the motility of their eupyrene sperm is greater (He and Miyata 1997).

In the field cricket, *Teleogryllus oceanicus*, males can produce sperm of varying viability by altering the quantity of nutrients they allocate to sperm development (Thomas and Simmons 2007). Males can also assess female mating status by detecting the number of previously-inseminated ejaculates within the females reproductive tract in this species (Thomas and Simmons 2007). When one rival ejaculate is present, males increase the viability of the sperm they inseminate, whereas when two or more rival ejaculates are detected within females, males decrease the viability of the sperm they allocate (Thomas and Simmons 2007). Males react to the sperm competition risk represented by a single rival ejaculate by increasing investment, then react to the sperm competition intensity represented by several rival ejaculates by decreasing investment.

Sperm morphology can also be affected by post-copulatory sexual selection. Some studies have demonstrated an increase in sperm length (Gomendio and Roldán 1991), and an increase in volume of the mid-piece of sperm (Anderson and Dixson 2002), with increasing risk of sperm competition in primates and rodents, whereas other have failed to find any relationship, once phylogeny is controlled for (Gage and Freckleton 2003). In butterflies and moths, the

length of eupyrene sperm has been shown to increase with measures of sperm competition (Gage 1994; Morrow and Gage 2000), and similar relationships have been found in cichlids (Balshine *et al* 2001) and birds (Johnson and Briskie 1999). In these instances, the greater degree of post-copulatory sexual selection in some species has selected for increased sperm length over evolutionary time; males are unlikely to be able to control the length of the sperm they produce. It has been hypothesised that longer sperm are faster swimmers within the female reproductive tract, so might achieve greater success during sperm competition (Gomendio and Roldán 1991), but this has yet to be convincingly proven. In some fishes, it has been demonstrated that longer sperm persist for shorter periods of time (Gage *et al* 2002), but in this instance, sperm length does not appear to be related to sperm swimming speed (Gage *et al* 2002).

1.3.3. Altering non-sperm ejaculatory components

The effects of non-sperm ejaculatory components on female and, resultantly, male fitness means that, by altering their allocation in ejaculates, males can effectively react to post-copulatory sexual selection. Substances including water, nutrients, and accessory gland chemicals can in some species be differentially allocated depending on the potential reproductive returns of different matings. In addition, the volume of an ejaculate can affect male success under post-copulatory sexual selection. In the snow crab, *Callinectes opilio*, males increase the volume of ejaculate they allocate to matings with females that have mated multiple times previously, in attempt to physically displace sperm inseminated by rival males (Rondeau and Sainte-Marie 2001).

In some butterflies, males can differentially allocate the sperm and the nutritious component of their ejaculates. In the small white butterfly, *Pieris rapae*, males allocate their reproductive resources differently depending on the point of the mating season (Cook and Wedell 1996). During their first mating of the season, males inseminate few sperm but lots

of nutritional substances with their ejaculates, and during their second mating, males increase sperm numbers but reduce their allocation of nutrients (Cook and Wedell 1996). Early in the season, females are more likely to be virgins, so the risk of sperm competition is low and therefore few sperm are needed to successfully achieve fertilisation success. The resulting offspring are likely to belong to the focal male, so it is adaptive to invest nutritionally in them. Conversely, later in the mating season, females are likely to have already mated, so there is a greater risk of sperm competition, therefore it is adaptive to increase sperm numbers to more effectively engage in the competition. At the same time, some of the offspring females produce might belong to rival competitors, so males reduce their provisioning of nutrients to avoid investing in offspring that are not genetically their own (Cook and Wedell 1996; Wedell and Cook 1999). Post-copulatory sexual selection therefore selects for males that can strategically adjust their ejaculate allocation depending on female mating status in this species. Similarly, in the bushcricket, *Kawanaphila nartee*, males reduce their provisioning of nuptial gifts to large females (Simmons and Kvarnemo 1997). In this species, because of the benefits of receiving a nutritious spermatophylax, females compete to mate with males (courtship is role-reversed). Larger females are more successful at achieving copulations, therefore larger females mate with more males. Sperm competition is therefore much more intense when mating with large females, due to the large number of ejaculates with which a mating male must compete. It is adaptive in this case for males to reduce their allocation to large females because nutrients they provide would likely be invested in offspring fathered by rival males. By increasing the size of the spermatophylax allocated to smaller females, males invest in the fitness of their own offspring and therefore indirectly increase their own reproductive success.

In another cricket, *Requena verticalis*, males alter their allocation of both sperm and their nutritious spermatophylax depending on female age (Simmons *et al* 1993). When mating with young females, males inseminate few sperm and a large spermatophylax (containing more nutrition for females), and when mating with older females they inseminate more sperm

and a smaller spermatophylax (Simmons *et al* 1993). Older females are more likely to have previously mated with rivals, so the risk of facing sperm competition is increased; males allocate more sperm accordingly. But because the female will likely still produce offspring fathered by previously-mating rivals, males reduce their investment in nutrition, which can be converted into offspring fitness, to avoid investing in offspring belonging to other males (Simmons *et al* 1993). Younger females will more likely be virgins, so few sperm are needed to ensure paternity, due to a low risk of sperm competition, but males invest heavily in nutrient provisioning in attempt to increase the fitness of the offspring the female will produce (which will likely belong to him) (Simmons *et al* 1993).

The efficiency of mating plugs can also be controlled by males in some species. In blue crabs, *Callinectes sapidus*, when rivals are present, males allocate larger ejaculates and maintain longer post-copulatory associations with females (Jivoff 1997). This delays female re-mating, and gives constituents in the ejaculate time to harden into a solid plug (Jivoff 1997).

1.4. Plastic versus fixed differences

In species in which conditions in early life affect male resource acquisition, or in which males adopt different mating roles based on body size, it might be expected that male ejaculate allocation patterns could be fixed for all of adulthood. Conversely, in species in which adult conditions vary temporally or spatially, it might be expected that male ejaculate allocation patterns remain under plastic control in adult life. This is because of the different selection pressures acting in different situations. When, for example, males are reared under resource-limited conditions as larvae, they might have to trade off body growth and development against investment in reproduction. In order to achieve sufficient somatic growth and body size, males might reduce their investment in reproduction, and so emerge as adults with a lower reproductive potential than males that were not resource-limited during larval growth.

Males having faced trade-offs might therefore have limited ability to invest heavily in matings, so ejaculates might be small. Males reared under resource-unlimited conditions might have had little need to trade off investment in reproduction against somatic growth, so might emerge as adults with excess reproductive resources; therefore ejaculates produced by these males might be relatively large. Evidence supporting these effects of early life conditions on male ejaculate allocation patterns includes findings in the adzuki bean beetle, *Callosobruchus chinensis*, in which males of monandrous strains reared with competition for resources produce fewer sperm than males reared without resource competition (Yamane and Miyatake 2008). In this example, however, the strain-specific mating system means it is likely males have not been selected to react to post-copulatory sexual selection, so are unlikely to perceive, and react to, levels of sperm condition indicated by larval density. In *C. chinensis* strains with polyandrous mating systems, males reared at high larval densities actually produce more sperm than those reared at low densities (Yamane and Miyatake 2005; 2008), thus appearing to overcome resource limitation and invest highly in reproduction instead of investing in somatic growth (males from high larval densities emerged smaller). It is as yet unclear whether males from any strains retain plasticity of ejaculate allocation as adults, or whether allocation patterns are fixed for life as a result of larval conditions.

In *Plodia interpunctella* moths, males given insufficient nutrition (only bran and glycerol) as larvae produce fewer sperm as adults than males given a richer early diet (yeast as well and bran and glycerol) (Gage and Cook 1994), again highlighting the effects of developmental trade-offs on adult ejaculate allocation. In this case, the differences in ejaculate allocations between males subject to different early-life conditions might be fixed for life, although other studies in this species have demonstrated males have the ability to allocate ejaculates plastically based on female age (Cook and Gage 1995) - whether males retain this plasticity of behaviour despite harsh larval conditions requires further investigation. It might actually be expected that, having more limited resources due to unfavourable early conditions, males might be even more strongly selected to exhibit sperm economy as adults; whether this is the

case, or whether harsh larval conditions put fixed limits on ejaculate allocation for life, is as yet unknown.

Ejaculate allocation strategies can sometimes be fixed in species in which males have different roles. In the Atlantic salmon, *Salmo salar*, small sneaker males allocate more sperm to matings, relative to their body sizes, than do large guarding males (Gage *et al* 1995), because small males are subject to high levels of sperm competition, as the females they sneak matings with also mate with their guarding male partners.

In contrast, in some species, the changing levels of post-copulatory sexual selection in adulthood is a stronger force, selecting males to be able to detect and react to the potential reproductive value of different matings, and allocate their ejaculates accordingly. There is evidence of this in numerous insects. Males of the mealworm beetle, *Tenebrio molitor*, vary the number of sperm they allocate to matings depending on their socio-sexual surroundings (Gage and Baker 1991); when mating in the presence of a rival, males allocate more sperm to their ejaculates than when mating with no rivals present (Gage and Baker 1991). This alteration of allocation occurs even when males are in the presence of rivals for only five minutes prior to copulation (Gage and Baker 1991), suggesting short-term plastic control of sperm number in this species. Similarly, in the egg parasitoid, *Trichogramma turkestanica*, males alter sperm allocation depending on rival male presence (Martel *et al* 2008); males kept alone inseminate more sperm than males kept with rivals (Martel *et al* 2008), due to the increased intensity of sperm competition in the latter situation making it adaptive to save sperm for more fruitful future mating opportunities.

1.5. Ejaculate adjustment and male fitness

Despite lots of studies showing changes in ejaculate adjustment that seem to make adaptive sense, there is a scarcity of information taking it right through to consequent effects on male

fitness. In a recent review on sperm competition, Bretman *et al* (2011) identified the fitness consequences of ejaculate modification as a fruitful course for investigation; this thesis aims to begin to address this using a model insect.

In a recent study using *Drosophila melanogaster*, Bretman *et al* (2009) found that males exposed to higher risks of sperm competition increase investment in ejaculate, and as a result these males achieve greater reproductive success than males exposed to lower risks of sperm competition, that invest less in ejaculate (Bretman *et al* 2009). This suggests the plasticity in ejaculate allocation is in this case an adaptive behaviour with demonstrable fitness consequences. However, males exposed to a higher risk of sperm competition (experiencing rival male presence) were kept in groups during copulations with the allocated female - it is therefore likely that the male achieving mating would be the strongest male of the group, so, compared to males exposed to a lower risk of sperm competition (remaining solitary), these males are likely to be of a more highly selected subset. It is unsurprising such males would achieve greater reproductive success. Studies are therefore needed in which male reproductive success depending on only ejaculate allocation is investigated, without any other confounding effects of male quality, before it can be properly determined whether plastic ejaculate allocation behaviours are indeed adaptive.

In another recent study using several species of *Drosophila*, Lizé *et al* (2012) discovered that males of species with monogamous mating systems surprisingly reacted to an increased risk of sperm competition by increasing mating effort (Lizé *et al* 2012). This is unexpected, given that the level of post-copulatory sexual selection acting on males of these species would be weak, since females do not mate multiply, so males would not be expected to be selected to react plastically to sperm competition level. This leads to questions about the adaptive nature of plasticity of ejaculate allocation. It is suggested either that plasticity is still selected for, despite very low levels of female re-mating in these species; that although plasticity is no longer adaptive, it might have been in the recent evolutionary past; that plasticity is adaptive

in monandrous mating systems, but for some reason other than post-copulatory sexual selection; or simply that the behaviour is not adaptive (Lizé *et al* 2012). These findings give even more strength to the argument that it cannot always be assumed that plasticity of ejaculate allocation is adaptive. More studies are required in which the fitness consequences of plastic ejaculate allocation are investigated, before it can be reliably determined whether the behaviour is adaptive.

In this thesis, I will examine directly the effects of ejaculate allocation on male fitness in the bruchid beetle, *Callosobruchus maculatus*. First, I examine how different types of social environment at different stages of a male's life can affect allocation decisions, and secondly, and most importantly, assess the consequences these have for actual male fitness under the normal competitive situations they would face - only this way can it be established if the effects are actually adaptive, and not simply a consequence of basic life-history trade-offs.

1.6. *Callosobruchus maculatus*

Callosobruchus maculatus beetles are a widespread pest of stored legumes in tropical and sub-tropical areas. They are suitable insects for investigation into sexual selection because females mate polyandrously and store ejaculates from multiple males in their spermathecae (Eady 1994; 1995). Because *C. maculatus* cause such significant damage to grain stores, the study of causes and consequences of adaptations to post-copulatory sexual selection in this species could prove useful in controlling population sizes, and could potentially help limit the economical damage inflicted on farms in tropical areas.

C. maculatus populations can change quickly in density; when a grain store is first colonised, density will be low and pioneering adults will mate and lay eggs, which will then hatch in large batches a number of weeks later. This process of hatching and death leads to boom and bust population dynamics; consequently, encounter rates of individuals vary over time and

with generations, therefore males can experience different levels of sperm competition over their lifetimes, as their socio-sexual surroundings change. It can be expected, therefore, that plastic ejaculate allocation ability in males might well be important in this species.

Females lay eggs on the outsides of various dried seeds and beans, and larvae hatch into the insides of beans, where they develop. Adults then emerge through holes chewed in the bean casing, and are able to mate immediately. Adults require neither food nor water, and are thus suited to the dry storage conditions in which they are often found. On their intrommitent organs, males possess hard spines that, during copulation, damage the female reproductive tract and might also allow males to prolong copulation, due to anchorage within the female (Crudgington and Siva-Jothy 2000). Male *C. maculatus* typically inseminate very high numbers of sperm; 85 % more than can be contained within the female spermatheca (most excess sperm remain in the bursa copulatrix) (Eady 1994; 1995). When mating, males transfer sperm via a spermatophore, which is inserted into the bursa copulatrix of the female (Eady 1994). Copulation has to last for two to three minutes before sperm are transferred, and after a further few minutes sperm move into the spermatheca (Eady *et al* 2004). Many of the excess sperm inseminated remain in the bursa copulatrix and are degraded, and possibly metabolised by females for energy used for egg production (Eady 1994). Following insemination, sperm are passively lost from the spermatheca at a constant rate (Eady 1994; Eady *et al* 2004).

The effects of sexual selection on *C. maculatus* have been widely studied. When two males mate with the same female, the second male fathers around 83 % of the offspring that a female subsequently produces (Eady 1994); *C. maculatus* therefore exhibit strong last-male precedence. The mechanism of sperm competition in *C. maculatus* has been identified as indirect sperm displacement (Eady 1995). This displacement might mean direct sperm competition can sometimes be avoided if two ejaculates are not in direct competition. In this case, we might expect selection not to act on sperm themselves but rather on male

behavioural or morphological characteristics that increase the likelihood of being in the correct mating position, or on other attributes of the ejaculate that might increase its ability to displace rival inseminations. However, the fact that displacement is incomplete (Eady 1995) and that females lay broods of eggs of mixed parentage, suggests that, at least sometimes, sperm from rival males are indeed under direct competition for fertilisations. Therefore, sexual selection will act on the sperm themselves as well as other ejaculatory components. That males inseminate so many more sperm than can be effectively contained within females (Eady 1994) supports this, because the fact that males have been selected to produce vast numbers of sperm suggests it might function to improve male reproductive success. Indeed previous work on *C. maculatus* has found sperm number to be important in determining male fitness (Eady 1995). Eady found that when the number of sperm transferred by the last-mating male was reduced, last-male fertilisation precedence decreased (Eady 1995). It might therefore be expected that males would react to post-copulatory sexual selection by increasing the number of sperm in their ejaculate, to maximise their own fertilisation precedence.

Because, in some insects, sperm displacement occurs due to the movement of two rival ejaculates relative to each other within the female reproductive tract, the occurrence of sperm displacement in *C. maculatus* means sexual selection might also be expected to act on ejaculate volume, because a larger ejaculate might more effectively displace a previously-inseminated ejaculate, or might decrease the chance of a male's own ejaculate being displaced by a subsequently-inseminated ejaculate. This might happen if inseminating a large ejaculate prevents a female re-mating again immediately, or postpones the point at which she will next accept another mating. Indeed it has been found that larger ejaculates delay female re-mating in *C. maculatus* (Eady 1995), suggesting males have been selected to increase the volumes of their ejaculates, to increase the chance of avoiding sperm competition by preventing or delaying the insemination of a subsequent ejaculate by a rival.

The ecology of *C. maculatus* could also provide clues about the sorts of reproductive resource allocation patterns that males might exhibit. Because both males and females live for short periods of time, and both sexes mate multiply (Ofuya 1995), any adaptations in males that increase longevity of both themselves and their female mates, increase the number of mating opportunities they have, or decrease the likelihood that their female mates will re-mate subsequently, could improve male lifetime reproductive success, and therefore would be expected to have been selected for. Because they live in arid climates and obtain all their lifetime resources during larval growth within bean hosts (Savalli and Fox 1999), any adaptation increasing the quantity of resources individuals are able to obtain might increase their longevity, and therefore increase the number of mating opportunities they have. It is also possible that, if males are able to obtain more resources, they might be able to allocate some of their extra resources to female mates via their ejaculates when they mate. By providing females with additional resources, the females might also live longer and so might have more time to lay more eggs, therefore male reproductive success might increase by proxy. For females, gaining resources such as nutrients might give them more energy to lay more eggs, and offspring resulting from these eggs might be fitter. Males could therefore also benefit from increased quality of offspring if they provide more resources for their female mates. It has been suggested that *C. maculatus* females might re-mate to obtain resources (Edvardsson 2007); if this is the case, by increasing their allocation of resources to females during mating, males might decrease the likelihood of females mating subsequently with rivals, therefore decreasing the likelihood of facing sperm competition and losing paternity to competitors. Adaptations that might prolong female longevity and decrease female receptivity to re-mating include increasing ejaculate size, and increasing the provision of nutrients within ejaculates.

In the wild, *C. maculatus* individuals experience hot and dry conditions, and access to water is limited; any adaptations increasing the availability of water for both males and their female mates might increase fitness in both sexes. Unless water reserves are available during adult

life, the only opportunity individuals have to obtain it is during larval growth within beans. Therefore, the only opportunities females have to get water as adults are via the ejaculates they receive during copulation. Adaptations by males that increase the water provision in their ejaculates might increase female fitness in the same way as nutrient provision; females that are more hydrated might live longer, lay more eggs and produce fitter offspring. There is evidence that female *C. maculatus* provided with water as adults do have increased longevity and fecundity, and also re-mate less frequently, than females denied water (Edvardsson 2007); if these same effects occur when females gain water via ejaculates, then males might be selected to maximise the water content of their inseminations.

In any population of *C. maculatus*, females might differ in their level of hydration and nutrition; since males are limited in the quantity of resources they have, adaptations enabling them to detect female condition might allow them to allocate their resources economically to different females. Males might therefore be expected to have been selected to assess female condition before inseminating their ejaculates. In some species, female condition is proportional to body size (Rasotto and Shapiro 1998) or abdomen size (Gage 1998).

Despite these adaptations that might increase male reproductive success in *C. maculatus*, males might have limited ability to adopt them if their own resource uptake is limited during larval growth. Large numbers of *C. maculatus* larvae can develop within the same bean host; as many as 12 individuals have been found to emerge from a single black-eyed bean host (Giga and Smith 1991) - due to competition for resource acquisition, males developing in beans with lots of other larvae are likely to be more resource-limited than males developing solitarily in beans. Larval conditions might therefore set limits on how reactive males can be as adults, in terms of attempting to increase their reproductive success. In any population of *C. maculatus*, both fixed and flexible patterns of ejaculate allocation might be expected to be seen. In males that experience tough larval conditions, resource limitation might limit their maximal ejaculate allocation, and might render them unable to effectively tailor allocations

differently to matings with different females. In this case, we might see set patterns of ejaculate allocation among males - those reared with competition for resources producing consistently smaller ejaculates than those reared without competition for resources. But because of the varying nature of adult socio-sexual conditions in *C. maculatus*, we might also expect to see plasticity of ejaculate allocation among males in a population, and within individual males across different matings. Males experiencing a high degree of competition for mates as adults might increase their ejaculate allocation to increase their chances of success under post-copulatory sexual selection, while males experiencing no adult competition might decrease their allocation, due to the lack of post-copulatory sexual selection. Despite a wealth of studies in *C. maculatus*, it is as yet unclear which of these selective forces acts more strongly on males - whether larval conditions fix male performance for life, or whether the highly varying level of polyandry selects for plasticity of ejaculate allocation, or a combination of both.

Findings in related beetle species could give clues about the strength of different selective forces. In the adzuki bean beetle *C. chinensis*, which is closely related to *C. maculatus*, studies have revealed larval conditions to be more formative to male ejaculate allocation patterns than adult conditions (Yamane and Miyatake 2005; 2008). Whereas altering adult socio-sexual circumstances has no effect on male ejaculate allocation (Yamane and Miyatake 2005), manipulating larval conditions does affect ejaculate allocation. In highly polyandrous strains, males reared at high larval densities produce more sperm than males reared at low larval densities (Yamane and Miyatake 2005; 2008). This is surprising, considering individuals reared at high densities would be expected to be resource-limited; that they still produce more sperm suggests males are under strong selection to engage in sperm competition by increasing sperm numbers. In monandrous strains, males reared at high densities produce fewer sperm than those reared at low densities (Yamane and Miyatake 2008); this suggests in this case ecology limits the reproductive ability of males and, in contrast to males of polyandrous strains, these males have not been selected to react to larval

density as a cue for sperm competition level. *C. maculatus* populations generally mate polyandrously; it might therefore be expected that male *C. maculatus* might follow the same pattern of ejaculate allocation as do male *C. chinensis* from polyandrous strains - producing more sperm when reared with larval competition for resources, but not reacting to adult socio-sexual circumstances. Yamane and Miyatake suggest the lack of effect of adult competitor presence on male sperm allocation can be explained by the period of non-receptivity to re-mating occurring in females after they have mated; therefore rendering current socio-sexual cues of no use for predicting sperm competition level. *C. maculatus* females have a similar life-history; generally being non-receptive to re-mating for a time after an initial copulation. It might therefore be expected that adult socio-sexual circumstances do not reliably predict sperm competition level in *C. maculatus*, so males might not have been selected to react to this. However, because Yamane and Miyatake only took sperm into account, and in *C. maculatus* ejaculate volume as a whole (not just the sperm component) has been shown to be important (Eady 1994; 1995), adult conditions could still affect male ejaculate allocation patterns, even if sperm themselves are not involved.

It is evident from previous work on *C. maculatus* that males are selected through post-copulatory sexual selection to maximise the numbers of sperm in their inseminations and the volumes of their ejaculates, in order to more effectively avoid, and engage in, sperm competition (Eady 1994; 1995). However, males only have a finite supply of reproductive resources that they must divide between several matings in their lifetimes. The limited nature of ejaculatory resources in *C. maculatus* is evidenced by the fact that ejaculate size decreases with each insemination when males mate sequentially (Savalli and Fox 1999). The fact that socio-sexual conditions change over the course of an individual male's lifetime, coupled with the evidence that males are selected to increase the numbers of sperm they inseminate and the sizes of their ejaculates, and the findings that doing so increases their reproductive success (Eady 1994; 1995), suggests the way males divide their reproductive resources between different matings during their lifetimes is of crucial importance in determining their lifetime

reproductive success. Although it might be advantageous for males to increase their reproductive resource allocation to each mating in the short term, changing conditions and differing reproductive potentials of different matings might mean in some instances it is beneficial to reduce allocation, in order to exhibit economical use of limited resources. Because males mate multiply and have finite reproductive resources, it is expected that *C. maculatus* males will have been selected to be able to detect the relative worth of different matings, and the likelihood that their ejaculates will have to compete with those of rivals. This thesis aims to begin to understand the way in which male *C. maculatus* might divide their ejaculate supply over matings occurring under different circumstances, and to investigate what cues males might use to decide on the size of their allocation. I examine how males differ in their ejaculate allocation when faced with different socio-sexual surroundings, and also investigate whether limiting conditions during early life put limits on the reproductive ability of males. Importantly, and unlike many studies using other insects, I examine the fitness consequences of different ejaculate allocation tactics, to determine whether apparent attempts by males to exhibit sperm economy does actually improve their reproductive success. This is important in establishing whether plastic ejaculate allocation is adaptive, and is potentially a trait that could be selected for in this species.

In numerous insect studies, it has been demonstrated that males alter ejaculate allocation depending on various conditions, and in some different studies the benefits of doing so, in terms of male lifetime reproductive success, have been investigated. However, few studies have linked these two stages by directly measuring both ejaculate adjustment in response to different conditions, and consequent fitness benefits (but see Bretman *et al* 2009; Tomkins and Simmons 2000; McNamara *et al* 2009). By using *C. maculatus* to do just that, I hope my findings might add to the field of evidence of causes and consequences of ejaculate allocation, and might even shed light on patterns found in other insects.

1.7. Thesis aims and outline

This thesis aims to investigate causes and consequences of ejaculate allocation in male *Callosobruchus maculatus*. I aim to identify some of the factors leading to differential allocation of ejaculate, and to establish whether different allocations have fitness consequences for males.

In Chapter 3, I examine whether adult social context affects ejaculate size in this species, and find that males increase the sizes of their ejaculates when exposed to rival male presence. This behaviour, however, is unexpectedly found not to increase male reproductive success.

Chapter 4 goes on to investigate whether conditions at a different stage of life - larval development - affect male ejaculate allocation, and finds that males reared at high larval densities produce smaller ejaculates than those reared at low larval densities. I also measure consequent fitness levels and find that, this time, males allocating larger ejaculates do achieve greater reproductive success. In Chapter 5, I investigate effects of larval conditions further, by examining effects on sperm number. I find that males reared at high larval densities produce fewer sperm than those reared at low densities. In addition, I find that high density males are less able than low density males to fertilise a clutch of eggs after several sequential matings.

Finally, in Chapter 6, I investigate the effect of providing males and females with water on ejaculate size, and find that males given water produce larger ejaculates, and females given water receive smaller ejaculates. My thesis as a whole provides evidence that various life-history factors can influence ejaculate size in *C. maculatus*, but suggests not all changes in ejaculate allocation have fitness consequences for males.

Chapter 2. Material and methods

This chapter outlines the general methodology used throughout the thesis and presents pilot data underlying some of the methods used. Each data chapter contains its own methods section detailing relevant experiments; this chapter is included to provide background information on more general materials and laboratory protocols.

Throughout my thesis I use the bean beetle *Callosobruchus maculatus* as a model organism for investigating post-copulatory sexual selection, as females mate polyandrously and store sperm internally (Eady 1994). *C. maculatus* are of the family Bruchidae and, along with other species of the *Callosobruchus* genus, are pests of stored grain products in tropical and sub-tropical areas, including parts of South America, Africa, Asia, Australasia and Europe (Southgate 1979).

2.1. Population details

Two strains of *C. maculatus* were used in the experiments; the Campinas strain from Brazil and the Niamey strain from Niger. The Campinas strain is of the same origin as that used by Credland (1986); it was collected from Brazil in 1974 and was maintained as a stock culture at Imperial College, London, from 1984. This strain has been maintained at the University of Edinburgh since 2002. The Niamey strain derives from stock culture at the University of Lincoln and is of the same origin as that used by Eady (1991). It has been cultured at the University of Edinburgh since 2009.

2.2. Stock culture maintenance

Both strains were cultured in an insectary at temperatures of between 28 and 30 °C and with a 12: 12 hour light: dark cycle. All stock beetles were cultured on black-eyed beans; some

experiments were carried out in which individuals developed in mung beans as well (details can be found in methods sections of chapters). Experimental manipulations, matings and data collection (egg counts, offspring observations and sperm counts) were carried out at ambient room temperature, around 20 °C.

Both strains of *C. maculatus* were maintained using the same weekly culturing regime. Once weekly, around 200 adult beetles (both males and females) were taken from the culture that had been established four weeks previously. Beetles were anaesthetised using carbon dioxide gas applied through ventilation holes in the culture box; this rendered all live adults in the box temporarily unconscious so they could be accessed and moved easily. Beetles were separated from their bean substrate using a metal sieve; holes were large enough to allow beetles through but beans were kept in the box. Adults collected would generally be between zero and seven days old, so would be at suitable age to both mate and lay eggs. These adults were then added to a new box containing around 1000 new black-eyed beans, and were allowed to mate and oviposit until death; this would populate the new beans with larvae, which would then develop and emerge around three to four weeks later. As adults these individuals could themselves be used to set up future culture boxes.

By repeating this process weekly there was a rolling stock of the population; at any one time there would be one box containing adults that had emerged within the previous week, a second box containing individuals about to emerge as adults, a third box containing developing larvae that would emerge as adults the following week, a fourth box containing less-developed larvae that would emerge in two weeks time and a fifth box, newly set up, containing ovipositing adults and newly-laid eggs, which would develop and emerge as adults in three weeks time. This set-up meant there was always a supply of adults of between one and seven days of age for use in experiments.

2.3. General methods

2.3.1. Sexing adults

Adult *C. maculatus* are sexually dimorphic and can be reliably identified as male or female (Southgate *et al* 1957). Females tend to be larger and have elongated abdomens, particularly in early life, whereas males tend to be smaller with more blunt-ended abdomens. Sex is more easily identifiable in the Campinas strain, as adults tend to be sexually dimorphic in colour too - females are darker in colour and have markings on their elytra whereas males are paler brown and generally have plain elytra. In the Niamey strain, both sexes are black in colour, but the body size and shape dimorphism still allow sex to be reliably identified.

2.3.2. Anaesthesia technique

In order to easily work with adult beetles there is sometimes a need to temporarily anaesthetise them. This is done using carbon dioxide gas, which is introduced either through ventilation holes into a stock box when anaesthetising a large number of beetles, or into Petri dishes or Eppendorf tubes when individuals are being anaesthetised. The time taken for anaesthesia to take effect depends on the container being used and the density of beetles and beans in it. For individuals being anaesthetised in small Petri dishes or Eppendorf tubes, around 30 seconds exposure to carbon dioxide is generally enough to render them unconscious for sufficient time to, for example, weigh an individual. To anaesthetise an entire stock box, for example in order to get a large number of individuals to set up a new culture, around four minutes exposure to carbon dioxide gas is required to be able to comfortably access and move lots of beetles while they remain unconscious. Recovery from anaesthesia is quick in *C. maculatus*; beetles generally regain consciousness within a few minutes. It has been previously found in *C. maculatus* that exposure to carbon dioxide gas causes reduced female fitness (Dawson 1995); therefore in experiments all focal individuals

were anaesthetised the same number of times, and for the same duration each time, to standardise experience and to control for effects of anaesthesia.

2.3.3. Collecting virgins from stock population

All experiments required the collection of virgin males and females from the stock population. To do this, the box from the culture population containing beetles at the correct stage was identified - this was the one in which larvae had fully developed and would imminently emerge as adults. Beans containing fully-developed individuals could be identified visually by the appearance of a dark substance under the testa of the beans; these dark spots were adult beetles that would soon emerge. Such beans were removed from stock boxes and placed individually in 1.5 ml Eppendorf tubes, with ventilation holes in the lids. Beans were checked daily for adult emergence. On emergence, individuals were sexed and each given an individual identification number, and were placed individually in 55 mm Petri dishes until their use in the experiment.

Because most beans in stock population contained multiple larvae, beans were continually checked daily, to collect all virgin adults. In the case that multiple adults of opposing sexes emerged simultaneously, these individuals were removed and excluded from experiments as their mating status could not be known.

2.3.4. Mating diagnostics

Copulation tends to follow a set process in *C. maculatus*; males locate and chase females until they are able to mount them, then insert their intromittent organs into the female reproductive tract. Males then remain lodged within females for a number of minutes (generally between two and 25 minutes; personal observation) until they are removed by female kicking. Females can begin kicking males with their back legs some time before males are eventually

dislodged, at which time the male's intromittent organ becomes detached from the female reproductive tract. The exact start and end points of mating can be ambiguous; sometimes males attempt mounting a few times before successful copulation occurs. To ensure all pairs used in experiments had actually mated, all pairs were observed once introduced and mating was only determined to have taken place once males had mounted and had become securely lodged within the female, the female was stood still and the pair were connected for at least 30 seconds. The majority of pairs did successfully mate quickly once introduced. Mating was judged to have ended when the male and female were completely detached.

2.4. Ejaculate size measurements

To measure the sizes of ejaculates inseminated by males during mating, ejaculate weight was estimated by weighing females to the nearest 0.001 mg, using a microbalance, both before and after mating, and subtracting initial weight from final weight. Female weight gain was taken as proxy for ejaculate weight; Savalli and Fox (1999) found a strong positive correlation between male weight loss and female weight gain during mating, showing male allocation can be reliably estimated by measuring female ejaculate uptake. Female weight gain, rather than male weight loss, was chosen as the measurement of ejaculate weight in order to avoid negating by anaesthesia any potential effects of experimental treatments on male ejaculate allocation. In most of my experiments it was males rather than females that had their conditions manipulated so it was judged better to anaesthetise females.

2.4.1. Omission of certain individuals

On measuring ejaculate size by weighing females, any females having gained no weight, or having lost weight, during mating were excluded from experiments, as it was judged successful mating had not in fact occurred. Pilot work showed females that did not mate but that were left for ten minutes unmated, and weighed both at the beginning and the end of the

ten minute period, all lost weight (see Figure 2.1). It was therefore concluded that any gain in female weight did represent receipt of an ejaculate from a male, therefore all females gaining weight were included in analyses, as even small weight gains were likely to be due to insemination of ejaculates by males.

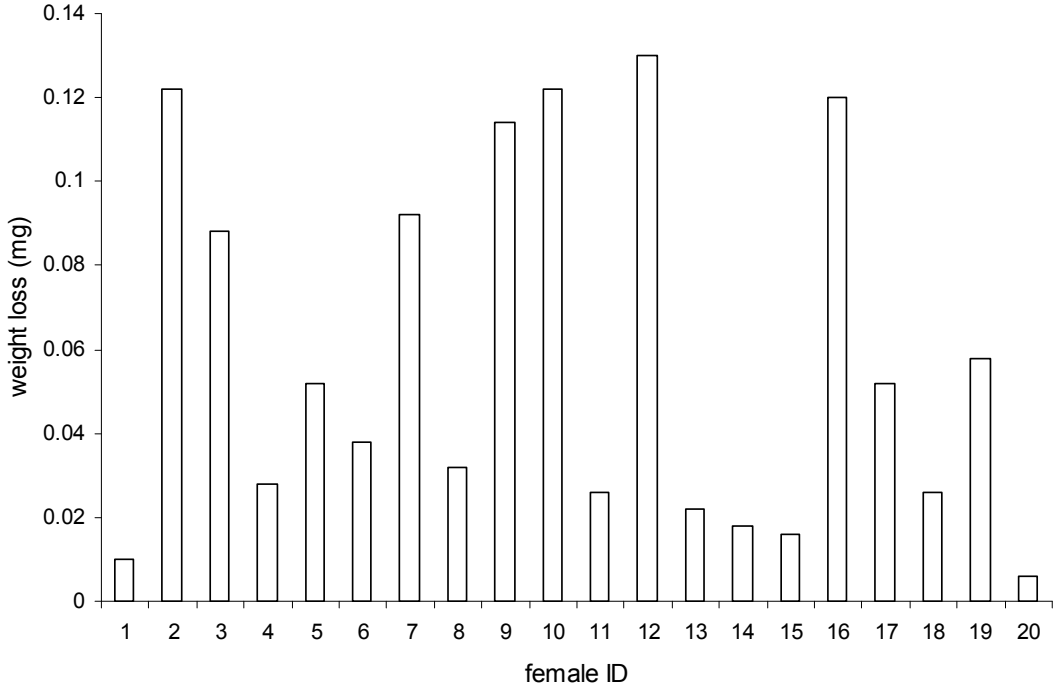

Figure 2.1: female weight loss in absence of mating. The weight losses of 20 females are given. Weight loss is given in milligrams. Since all females lost weight the bars represent negative weight changes.

2.5. Sterile male technique

The sterile male technique is a widely used method of paternity assignment in insects (Harano *et al* 2008; Eady 1991). This involves using gamma radiation to sterilise control males; sterilised males are still able to mate and their sperm can fertilise eggs, but DNA damage caused by exposure to radiation means fertilised eggs become inviable and do not develop into larvae. I used the sterile male technique to estimate reproductive success of focal males

by mating them competitively with females that also mated with sterile males. The gamma irradiator housed at the University of Edinburgh has Caesium 137 as its radioactive source. In order to determine the most effective dose of radiation, I carried out a pilot experiment, exposing groups of males to different doses (different times of exposure to Caesium 137). Virgin males were collected from stock culture of the Campinas strain of *C. maculatus* and were randomly allocated to one of seven radiation duration treatments; 0 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes and 9 minutes exposure. Within 30 minutes of exposure, males were mated to randomly allocated virgin females, also from Campinas stock culture, and copulations were observed. Following mating, males were removed and females were transferred to 90 mm Petri dishes containing around 100 black-eyed beans, and were allowed to oviposit until death. Five days following female death, beans were observed under a dissecting microscope and eggs were assigned as either hatched or un-hatched by their appearance. Hatched eggs had turned white, due to the burrowing action of larvae developing from viable fertilised eggs, whereas un-hatched eggs remained translucent, as they were unfertilised. The numbers of hatched and un-hatched eggs laid by each female were counted. Females having laid fertilised (hatched) eggs had mated with males whose sperm retained viability beyond just fertilising eggs; for use in experiments these males were unsuitable since eggs laid by females mating with two competitor males (one focal male and one sterile male) could not have their paternity assigned; some fertilised eggs might have been fathered by competitor males that were not completely sterilised. The numbers of fertilised (hatched) eggs laid by females mating with males exposed to gamma radiation for different durations can be seen in Figure 2.2.

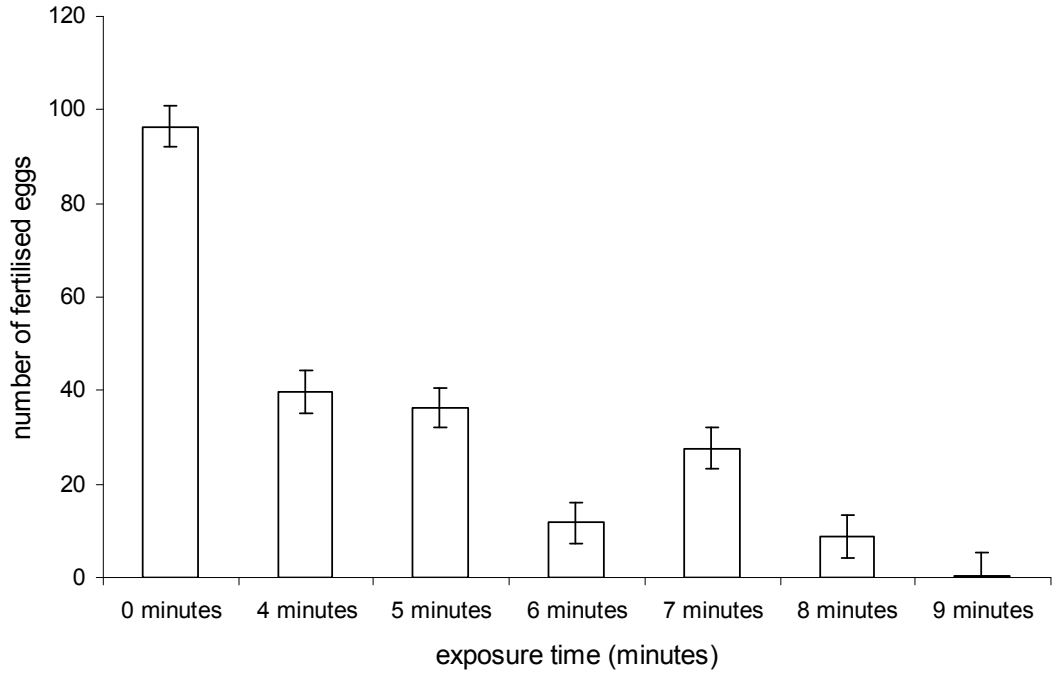

Figure 2.2: gamma radiation exposure and egg fertilisation. The number of fertilised eggs given is the mean value of groups of males having been exposed to each duration of radiation. Error bars show the standard error of the mean. Exposure time is given in minutes.

Nine minutes of exposure to radiation resulted in the most effective male sterility; males exposed to this duration (which equates to a 60 Gy dose of Caesium 137) produced almost zero viable eggs, yet they were still physically able to mate and their sperm could still fertilise eggs. Nine minutes (60 Gy) was therefore determined as the most suitable dose for use in the sterile male technique in this set of experiments - this maximised the efficacy of the sterilisation dose but minimised any risk of overexposure that might affect mating success.

2.6. Genetic markers

The second method of paternity assignment I used was genetic markers. The reproductive success of focal males was estimated by competing them with control males belonging to a strain carrying a genetic marker for black body colour; the Niamey strain (Eady 1991).

Females were first mated to focal Campinas strain males and then immediately offered a second mating with a black Niamey male. The females and their new black partners were left to mate whenever the females' natural period of latency elapsed. In some experiments, female receptivity was measured by separating eggs laid at different time-points, and in others females were left to oviposit on the same batch of beans until death (more details for these methods can be found in methods sections of the relevant chapters). In both cases, the method of paternity assignment by offspring appearance was the same. Offspring fathered by the first-mating brown (Campinas) male were wild-type brown in appearance, having both a brown mother and a brown father. Offspring fathered by the competitor black marker (Niamey) male had some brown and some black features, having a brown mother and a black father. By observing offspring under a dissecting microscope, paternity was assigned by the colour of particular body parts - the anterior leg-pair was the key diagnostic feature. In offspring fathered by the focal male the anterior legs were brown, whereas offspring fathered by the competitor male had black anterior legs. Offspring with black fathers tended to also be darker in other body areas including antennae, heads and elytra, but these were more variable. Paternity assignment by observation of the anterior leg pair was highly repeatable; pilot work showed results from two different observers were consistent in 60 out of 61 trials, therefore observational error was below 2 %.

2.7. Sperm counting

In Chapter 5, as well as measuring reproductive allocation by ejaculate weight, sperm counts were carried out. The protocol used was the same as that used by Eady (1995), and initial instruction and training was received from P E Eady and R Vasudev. Methods for counting sperm are detailed in Chapter 5, but during tuition sperm counts were found to be highly repeatable; counts of the same samples by two different experimenters were highly correlated (with a correlation of 0.994).

2.8. Statistical analyses

Data analyses were carried out using Minitab 15 and R. Details of specific analyses can be found in relevant chapters, but generally, where suitable, data were analysed using General Linear Models in Minitab. Residuals were checked for normality and, where needed, data were transformed in order to fit the assumptions of the models. Initially in models, all explanatory factors, covariates and their interactions were included to produce a maximal model; non-significant terms were then removed sequentially until the minimal model was obtained. Explanatory factors related to the experimental design (for example, block, mating order and bean type) were left in the minimal model even if they did not significantly affect the response variable, in order to soak up variation in the data to make potential effects of other variables clearer, and because leaving them in the models was more realistically representative of the data. For simplicity, in the results sections of chapters, p values of interactions are not reported unless they affect the response variable, or are of direct relevance to questions being asked.

Analyses carried out in R were Generalised Linear Models; these were used when analysing proportion data that could not reliably be analysed using General Linear Models in Minitab because data were not normally distributed. Models were fitted with quasibinomial errors; a model with binomial errors did not fit the data correctly because between-beetle variation meant the residual deviance was too high, and the quasibinomial error distribution fitted the data better. Again, all explanatory factors, covariates and their interactions were initially fitted to obtain the maximal model. Non-significant terms were removed sequentially and their effects tested using ANOVAs to compare the fit of the model with and without the relevant terms included. Minimal models contained any terms significantly affecting the response, and any factors related to experimental design. Again, for simplicity, effects of interactions are only reported if they significantly affect the response variable, or are of direct relevance to the questions being asked.

F statistics, chi-sq statistics and p values stated are all from minimal models in both types of analysis.

In Chapters 3 and 4, male reproductive success was measured in two ways; one of which was analysed in Minitab and the other in R. In most insect ejaculate allocation literature, male reproductive success is measured as P_2, the proportion of a clutch of offspring fathered by the second-mating male (or P_1, if the focal male is first to mate). To make my findings directly comparable with the literature, I measured this aspect of male reproductive success by analysing the proportion of a clutch fertilised, using Generalised Linear Models in R. However, I also considered the actual number of offspring produced (rather than the proportion of the clutch, which takes into account the relative reproductive success of the competitor male) to be an important measurement of male reproductive success. I therefore also analysed this, using General Linear Models in Minitab. The results of both types of analysis are reported, in attempt to properly and robustly investigate effects on male reproductive success.

Chapter 3. Ejaculate size adjustment in response to social context - is it always adaptive?

3.1. Introduction

The level of sperm competition has been shown to affect behavioural and physical attributes of males in numerous species; for example, males of species with polyandrous mating systems tend to have larger testes than closely related monogamous species (Gomendio *et al* 1998; Gage 1994; Hosken 1997; Stockley *et al* 1997; Hosken and Stockley 2004) due to heavier investment in sperm competition. In many situations, however, the competitive environment faced by an individual can change over time. The ability to alter a response to changing environmental conditions is key to being able to maximise success over an individual's lifetime. In such a situation, selection is likely to favour the ability of individuals to respond plastically, adjusting their behaviour depending on their current situation.

At first it would seem that, to succeed in sperm competition, males should maximise the sizes of their ejaculates under all circumstances. However, due to the finite nature of sperm (Wedell *et al* 2002) this is not always possible. In numerous species, sperm numbers and ejaculate allocation appear to be plastically controlled by males depending on anticipated levels of sperm competition, which can change temporally and spatially (Wedell and Cook 1999; Schaus and Sakaluk 2001; Danielsson 1998; Gage and Baker 1991; Nicholls *et al* 2001; Jivoff 1997; Pound and Gage 2004; Pizzari *et al* 2003; Martel *et al* 2008; Aspbury 2007). Such plasticity allows males to use their reproductive resources economically by increasing ejaculate size only when the risk of sperm competition is high (Schaus and Sakaluk 2001; Gage and Barnard 1996; Aspbury 2007). Often the purpose of this is to deliver more sperm to the female, in order to fertilise more eggs than do rival males (Engqvist *et al* 2007; Tomkins and Simmons 2000), such as in the cricket *Gryllus texensis* (Schaus and Sakaluk 2001). However, sometimes it is other ejaculatory components that are important. In cases

where males can detect whether females have mated before, ejaculatory substances which damage or kill rival sperm can be up-regulated, resulting in a larger ejaculate, as happens in some *Drosophila* species (Prout and Clark 2000), or the volume of a larger ejaculate might be advantageous in that it physically displaces rival sperm, as in the yellow dung fly (Simmons *et al* 1999). In other cases, when males mate with virgins or females without rival sperm in storage, they might add nutrients or water to their ejaculate to discourage females from re-mating subsequently (Fricke *et al* 2009; Rice 1996; Moreau *et al* 2002) by providing necessary resources, such as in Pieridae butterflies (Svärd and Wiklund 1989), or by up-regulating chemicals that enforce a refractory period in females, like in the hanging fly, *Bittacus apicalis* (Simmons and Siva-Jothy 1998); this can increase ejaculate size without increasing sperm number. In these situations, sperm competition might be avoided by preventing rival sperm having access to the ova set. How an ejaculate is altered in response to different levels of sperm competition will depend on whether males are able to detect and react to the risk, and whether it is sperm or other ejaculatory components that are more important for success in sperm competition. Ultimately these modifications are predicted to lead to greater reproductive success.

Despite many investigations into ejaculate allocation patterns with varying sperm competition risk in numerous different species (Gage and Baker 1991; Simmons *et al* 2007; Gage and Barnard 1996; Schaus and Sakaluk 2001; Cook and Gage 1995), few studies to date have investigated whether ejaculates of different sizes, or containing different sperm numbers, lead to the expected changes in male reproductive success. A recent study using *Drosophila melanogaster* (Bretman *et al* 2009) tested the response of males to the presence of rivals in terms of reproductive effort (copulation duration), and assessed whether increases in effort yielded increased male reproductive success; competitor presence prior to mating caused males to increase their mating effort, which increased their reproductive success (Bretman *et al* 2009). Unfortunately the design of this study makes it hard to draw strong conclusions about the consequences of ejaculate adjustment, as the males that mated in the "group"

treatment were not randomly selected. If males vary in their ability to compete for a female, and there is any relationship between the pre-copulatory and post-copulatory ability of males, this could potentially confound the results of the experiment.

Here, I use the polyandrous seed beetle *Callosobruchus maculatus* to test whether sperm competition affects male reproductive effort and success. Specifically, I investigate whether adjustment of ejaculate size occurs in response to different social surroundings. I examine the effects of the presence of rivals prior to mating on male ejaculate allocation and, importantly, test whether increased mating effort really does increase male fitness. *C. maculatus* males can experience varying levels of sperm competition over their lifetimes because, in their natural habitat of dried grain stores, population density can change rapidly as numbers decrease and increase as adults die off and new offspring hatch in large batches. Population sex ratio can also vary temporally as females tend to emerge slightly earlier than males (in my laboratory population); it is therefore expected that males of this species have been selected to react to varying socio-sexual circumstances.

In *C. maculatus*, increasing ejaculate size in response to sperm competition could be advantageous either because sperm numbers are increased, so males engage in sperm competition, or because non-sperm ejaculatory components are up-regulated, and females are discouraged from mating subsequently (Savalli and Fox 1999), so males avoid sperm competition. Eady (1994; 1995) showed that last male fertilisation precedence occurs in *C. maculatus* due to the displacement of previously-inseminated sperm by a new ejaculate. As a first-mating male, inseminating a larger ejaculate might therefore be advantageous because it delays female re-mating (Eady 1995), and as a last-mating male, a larger ejaculate might help increase paternity success by displacing sperm already inseminated by a rival (Eady 1994).

To manipulate perceived levels of sperm competition, I expose focal males to either a solitary or a group social context prior to mating. I use ejaculate weight as a measure of reproductive

effort, to take into account both the sperm and non-sperm components of the allocation. I then use two different methods of paternity assignment to assess whether alterations in male ejaculate allocation result in corresponding changes in male reproductive success. Based on sperm competition theory (Parker 1970), I predict that grouped *C. maculatus* males will increase their ejaculate allocation in response to the greater risk of sperm competition represented by the presence of their competitors. If this behaviour is adaptive, I also predict larger ejaculates to result in greater reproductive success.

3.2. Methods

3.2.1. Social context manipulation

A population of the Brazilian Campinas strain of *C. maculatus* was maintained on black-eyed beans (*Vigna unguiculata*) in an insectary heated to a temperature of 28 - 30 °C, with a 12 hour: 12 hour light: dark cycle. Beans containing developing larvae were removed from stock culture and isolated individually, to avoid adults mating on emergence. Within 24 hours of eclosion, individuals were sexed and given individual identification numbers. Newly emerged virgin males were left in individual dishes for 48 hours to allow full maturation of sperm stores (Savalli and Fox 1999) and were then randomly allocated to one of two treatments; solitary or group social context. Those males allocated to the solitary treatment remained alone in 55 mm Petri dishes for four hours. Males allocated to the group treatment were transferred into 55 mm dishes with four other males and left for four hours. The solitary treatment is designed to mimic an environment where the risk of sperm competition is low, since the focal male has no rivals in his surroundings, whilst in the group treatment, any or all of four rival males could potentially mate with a female should one become available; this was therefore designed to represent a greater risk of sperm competition. Social context manipulations similar to this have been shown to affect male mating behaviour in other insects (Gage and Baker 1991; Simmons *et al* 2007; Schaus and Sakaluk 2001).

Newly emerged females were collected, anaesthetised using carbon dioxide gas and weighed to the nearest 0.001 mg. Females were allowed to fully recover from anaesthesia (recovery is quick; within five minutes) before mating. All matings were carried out at ambient room temperature (~20 °C). Males in solitary or group treatments were randomly allocated to these females for mating. Solitary males were removed from their dishes and placed with their corresponding females, and copulation was observed. One of the five males in each group was randomly selected and placed with a female, and copulation was observed. The four remaining group males were discarded. Following mating, males and females were separated. Females were anaesthetised and re-weighed, and ejaculate size was calculated by subtracting weight before mating from weight after mating. Savalli and Fox (1999) found a strong positive correlation between male weight loss and female weight gain during mating, showing male allocation can be reliably approximated by measuring female ejaculate uptake; in my study I chose to measure female weight change to avoid negating by anaesthesia any potential effect of social context on male behaviour (Savalli and Fox, 1999). Any females found to have gained no weight (or to have lost weight) were assumed not to have mated and were therefore excluded from the study.

3.2.2. Measuring male reproductive success - paternity assignment

I estimated male reproductive success in a subset of males exposed to the two social contexts, using two different approaches: the sterile male technique (Harano *et al* 2008; Eady 1991) and genetic markers (Eady 1991), in order to compare the reproductive successes of males having experienced solitary and group social context treatments, by mating them under competitive circumstances.

The sterile male technique is a widely used method of determining paternity in insects (Harano *et al* 2008; Eady 1991). It involves mating a female with two males, one of which

has been sterilised (usually by exposure to ionising radiation). Paternity of a mixed clutch can then be estimated by comparing the numbers of fertilised and unfertilised eggs the female lays.

Competitor males were generated by collecting virgin males from the same culture as the focal males and irradiating them in groups, in accordance with the sterile male technique (Harano *et al* 2008) using Caesium 137 at a dose of 60 Gy (pilot work demonstrated this dose was the optimal exposure time to allow egg fertilisation but prevent offspring development; see Chapter 2 for further detail). New competitor males were irradiated daily throughout the experiment, and mated within one hour of irradiation, as the sterilising effects can wear off given sufficient time.

Following the initial mating (during which ejaculate size was measured) with a focal male, females (<24 hours of age) were given 20 black-eyed beans on which to lay eggs for 48 hours. Females were then re-mated to sterile males; copulation was observed and the twice-mated females were placed on 100 black-eyed beans and allowed to oviposit until death. Since ejaculate allocation might also depend on mating order, additional replicates were carried out with the reciprocal mating order. By doing this, it could be investigated whether males allocate ejaculate differently (and whether reproductive success is differently altered) depending on whether they mate before or after a competitor male.

Five days after the females died, all eggs that had been laid on beans were counted and assigned as either fertilised (hatched) or unfertilised (un-hatched) by their appearance and colour. In *C. maculatus*, larvae from fertilised eggs hatch out and burrow into the bean a few days after eggs have been laid, causing hatched eggs to change in appearance from pale and translucent to bright white. Eggs fertilised by sterile males fail to develop, so remain translucent. By allowing five days between female death and egg observation, it was ensured all eggs with the potential to hatch had done so. Therefore, all white eggs had been fertilised

by focal males, and translucent eggs had been fertilised by sterile males (natural levels of male fertility are very high in *C. maculatus*). The numbers of eggs fathered by each male in each competitive pair were counted, in order to determine whether males having experienced solitary and group treatments achieved different reproductive success when mating under competitive circumstances.

3.2.3. Measuring female receptivity

While the sterile male technique is a simple way of assigning paternity and estimating male reproductive success in insects, the fact that the time of the second mating is controlled by the experimenter means that any effect of ejaculate size via an effect on female receptivity will not be detected. Thus, I carried out an additional experimental block where paternity was assigned using genetic colour markers; a method of paternity assignment that allows females to re-mate after a natural period of non-receptivity. The fertilisation success of focal males was assessed by competing them against standardised control males of a different strain (the Niamey strain), which carried a genetic marker for black body colour (Eady 1991). New black males were collected daily as virgins and held individually until mating. Virgin focal males (brown morph) were subject to either solitary or group treatments following the methods described for the first experiment. These focal males were mated to virgin brown females (<24 hours of age), who were weighed before and after mating, giving a measurement of ejaculate size. Immediately following mating, the brown male was removed and the female was transferred along with a new black male to a dish containing 100 black-eyed beans. Every six hours for 54 hours following the initial mating, the female and black competitor male were transferred to a fresh dish of 100 black-eyed beans, in order to separate eggs laid during different time periods (0-6 hours, 6-12 hours, 12-18 hours, 18-24 hours, 24-30 hours, 30-36 hours, 36-42 hours, 42-48 hours and 48-54 hours). This allowed me to identify when second matings took place, to give a measure of 'latency to re-mate' following

copulation with males from different social context treatments. After 54 hours the male and female individuals were removed.

Dishes of beans were left for four weeks at around 30 °C; by this time offspring had emerged. Beans were removed and the offspring were left until death, after which time they were individually observed using a dissecting microscope and assigned as either black or brown. Individuals were categorised according to the colouring of their elytra, heads, antennae and legs. Brown individuals tended to be pale brown in all these areas; black individuals (with a brown mother and a black father) tended to have darker elytra and heads, and the first (anterior) leg pair was always black – this was the key diagnostic feature. This categorisation method was highly repeatable (see Chapter 2 for further detail).

Brown offspring had been fathered by the first, focal brown male, and the appearance of black offspring indicated that, at some point following the initial mating, the female had accepted a second mating from the black marker male. By examining in which dish black offspring first appeared, the time period during which the female re-mated could be determined. This gave a measurement of the period of female non-receptivity following an initial mating with a male from either a solitary or a group social context treatment. Unlike the previous subset, in which the sterile male technique was used, this time only one order of mating was carried out; focal males mated first. This was because in order for females to be allowed their natural period of latency before mating with a second male, the second mating could not be observed nor the second ejaculate size measured, so to get this information for the focal males they had to be first to mate.

For each female, numbers of brown and black offspring were counted. This allowed the reproductive success of focal males having experienced solitary and group social context treatments to be compared.

3.2.4. Statistical analyses

The experiment was run in six blocks. The initial step of ejaculate size measurement for males experiencing different social contexts was repeated in all six blocks. In later blocks, the two different methods of paternity assignment were used, to ensure results were robust. In blocks 3 to 5, the sterile male technique was used, then in block 6 genetic markers were used.

Analyses were carried out using Minitab 15 and R. When General Linear Models were used in Minitab, all explanatory factors and variables were fitted initially to produce the maximal model; non-significant terms were then removed one by one until the minimal model was obtained. The minimal model contained all factors that related to the experimental design (e.g. social context treatment, mating order and block), whether or not they had significant effects on the response variable. All stated statistics are from minimal models. Egg and offspring numbers (as a measurement of male reproductive success not taking into account paternity of competitor males) were analysed in Minitab; egg number and offspring number were square-root transformed in order to fit the assumptions of a normal distribution. Male paternity proportion data (egg and offspring counts relative to those achieved by competitor males) were analysed in R using Generalised Linear Models with quasibinomial errors (the quasibinomial error distribution was determined to be appropriate because between-beetle variation meant that residual deviance in a binomial distribution was too high). Again non-significant interactions were removed from the maximal model sequentially, and their significance tested using ANOVAs comparing the fit of the model with and without the term of interest.

The effects of all interactions were tested; for simplicity only those that had significant effects, or those that are related to questions being addressed, are reported.

3.3. Results

3.3.1. Social context and ejaculate size

Social context did affect ejaculate size; males that were grouped with rivals prior to mating inseminated ejaculates that were around 7.23 % larger than those that remained solitary ($F_{1, 403}$ = 6.12, p = 0.014), see Figure 3.1. Despite ejaculate size differing between experimental blocks ($F_{5, 398}$ = 6.32, p = 0.012), the effect of social context on ejaculate size was consistent across all blocks, as confirmed by the lack of effect of the interaction between social context and block ($F_{5, 393}$ = 0.36, p = 0.875).

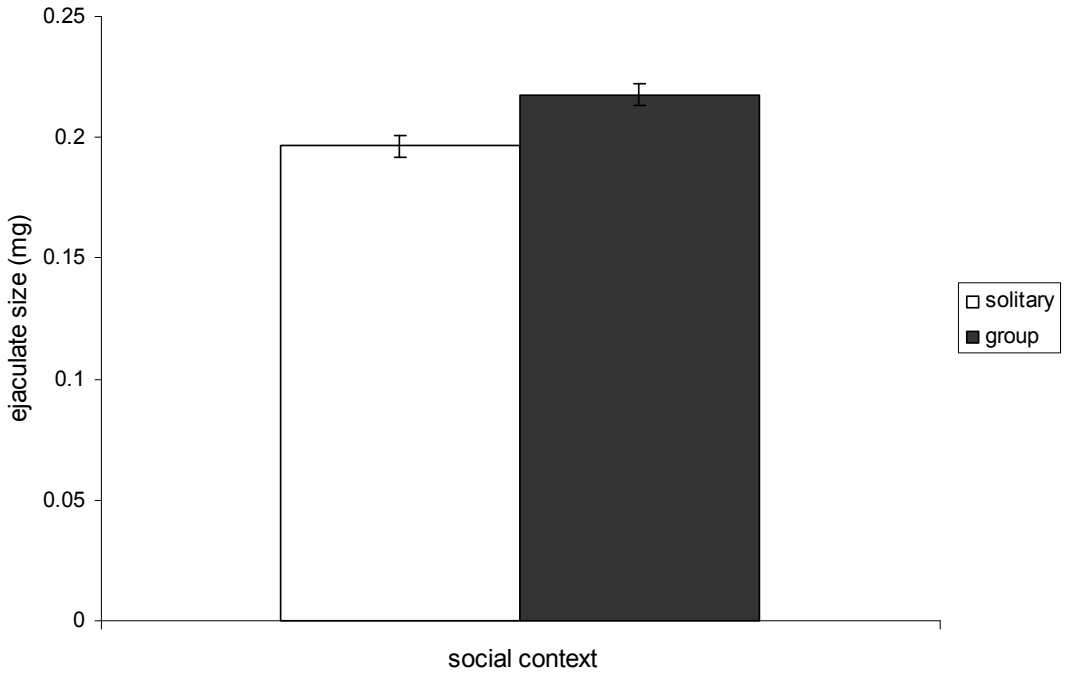

Figure 3.1: social context and ejaculate size. Ejaculate size is in milligrams, and is given as the mean value of males in each social context group. The empty bar represents males from the solitary treatment and the filled bar represents males from the group treatment. Error bars show the standard error of the mean. For solitary treatment males, n (sample size) = 208 and for group males, n = 197.

3.3.2. Social context and reproductive success

All analyses on reproductive success reported were done using full egg counts (including those laid between matings) rather than only those eggs laid after the second mating. Analyses including only those eggs laid after both matings were also carried out and yielded the same results; since results were unchanged regardless of which measure of reproductive success was used, only those using the full clutch are reported.

Despite the effect of social context on ejaculate size, social context did not affect male reproductive success, as measured by the number of eggs fertilised during competition (the sterile male technique). A general linear model was fitted with social context, mating order and block as explanatory factors, and the number of eggs fertilised as the response. The number of eggs fertilised by focal males was not affected by social context ($F_{1, 158} = 0.04$, $p = 0.839$); see Figure 3.2. Fertilised egg number was unaffected by mating order ($F_{1, 158} = 1.27$, $p = 0.262$) and although there was an effect of block ($F_{2, 158} = 8.07$, $p < 0.001$), there was no effect of the interaction between social context and block ($F_{2, 153} = 0.03$, $p = 0.973$); the effect of social context did not differ between blocks.

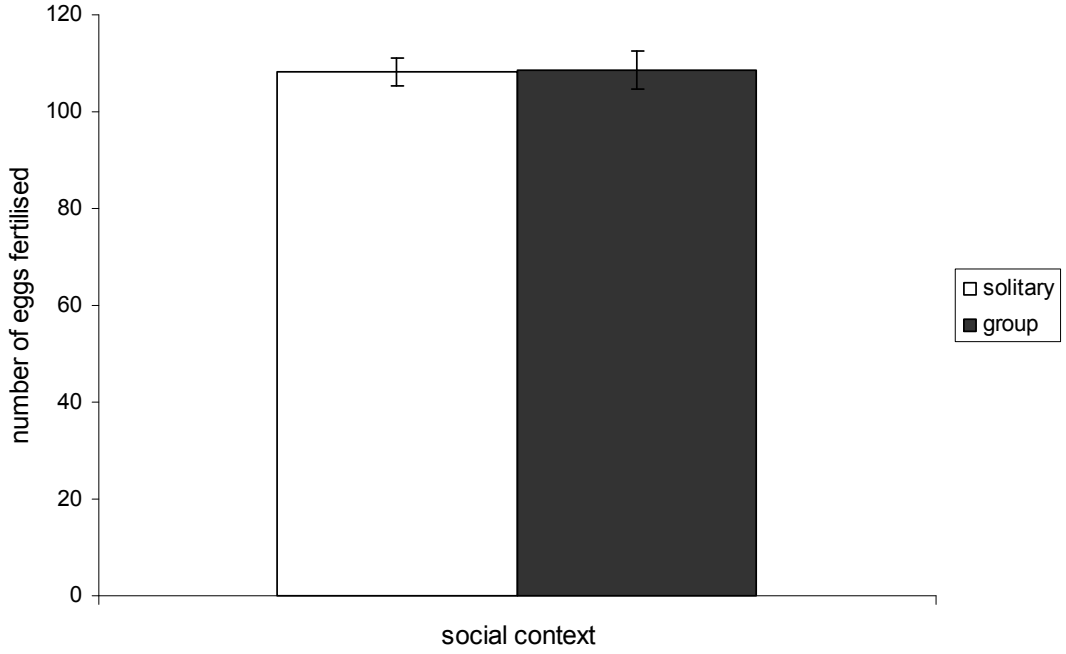

Figure 3.2: social context and fertilised egg number. Male reproductive success is given as the mean number of eggs fertilised by males in each social context treatment group. The empty bar represents males from the solitary treatment and the filled bar represents males from the group treatment. Error bars show the standard error of the mean. For solitary treatment males, n (sample size) = 84 and for group males, n = 79.

To examine whether there is any relationship between ejaculate size and number of eggs fertilised within social context treatments, the analysis was repeated but ejaculate size was added as a covariate. Ejaculate size did not affect fertilised egg number ($F_{1, 157} = 0.27$, $p = 0.604$), and controlling for it did not reveal any effect of social context ($F_{1, 157} = 0.05$, $p = 0.824$). Mating order did not affect fertilised egg number ($F_{1, 157} = 1.12$, $p = 0.291$), and although there was an effect of block ($F_{2, 157} = 8.13$, $p < 0.001$) there was no effect of the interaction between social context and block ($F_{2, 152} = 0.03$, $p = 0.969$); the effect of social context on fertilised egg number did not differ between blocks.

To take into account the reproductive success of focal males relative to that of their sterile competitors, a generalised linear model was conducted in R looking at the egg numbers of

both focal and competitor males, and having the proportion fertilised by the focal male as the response, with social context, mating order and block as explanatory factors. There was no effect of social context on proportion paternity (chi-sq = 0.36, df = 1, p = 0.764). Block did not affect proportion paternity (chi-sq = 10.22, df = 2, p = 0.474) but there was an effect of mating order (chi-sq = 1410.3, df = 1, $p < 0.001$) - males that mated first fertilised a greater proportion of the clutch than those that mated second (when the complete clutch was taken into account); see Figure 3.3. It has been previously demonstrated in this species that last-mating males have fertilisation precedence (Eady 1994); because in my experiment females had 48 hours between matings, during which time they laid large numbers of eggs, first-mating males have precedence. However, this does not bias the effect of social context in my results because the time between matings was the same in all females, whether they mated with solitary or group focal males. There was also an effect of the interaction between mating order and block (chi-sq = 502.45, df = 2, $p < 0.001$) on proportion paternity; although first-mating males always achieved greater paternity proportions than second-mating males, the size of the difference in paternity varied between blocks.

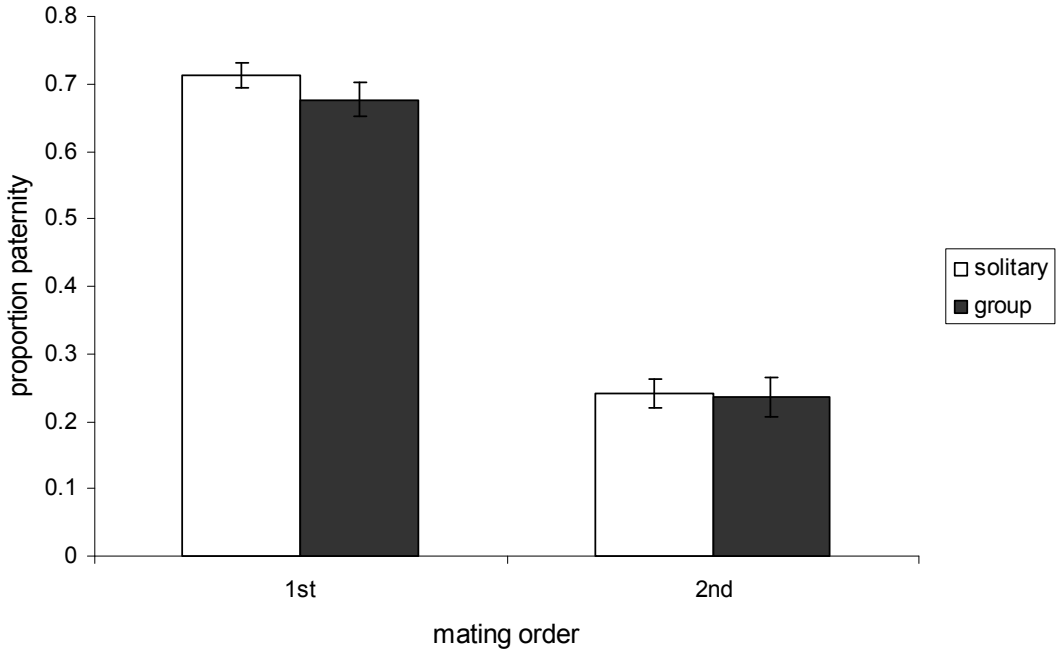

Figure 3.3: social context and proportion paternity. Male reproductive success is shown as proportion paternity of a clutch, and is given as the mean of males from each social context, and in both mating positions (first and second-mating males). Empty bars represent males from the solitary treatment and filled bars represent males from the group treatment. Error bars show the standard error of the mean. For solitary treatment males mating first, n (sample size) = 53, for solitary males mating second, n = 31, for group males mating first, n = 50 and for group males mating second, n = 29.

To examine potential associations between ejaculate size and paternity within social context treatments, the analysis was repeated but ejaculate size was added to the model as a covariate. Within a treatment, ejaculate size did affect the proportion of offspring sired by a male (chi-sq = 49.84, df = 1, p = 0.002); see Figure 3.4, but again despite controlling for this in the model, there was still no effect of social context on male reproductive success (chi-sq = 0.25, df = 1, p = 0.800). Block did not affect proportion paternity (chi-sq = 8.2, df = 2, p = 0.543) but again there was an effect of mating order (chi-sq = 1436.1, df = 1, $p < 0.001$) and of the interaction between mating order and block (chi-sq = 490.89, df = 2, $p < 0.001$); first-mating males always achieved greater paternity proportions than second-mating males, but the size of the difference varied between blocks. There was no effect of the interaction between social

context and ejaculate size on proportion paternity (chi-sq = 0.92, df = 1, p = 0.627); ejaculate size did not affect proportion paternity differently in the different social context treatments.

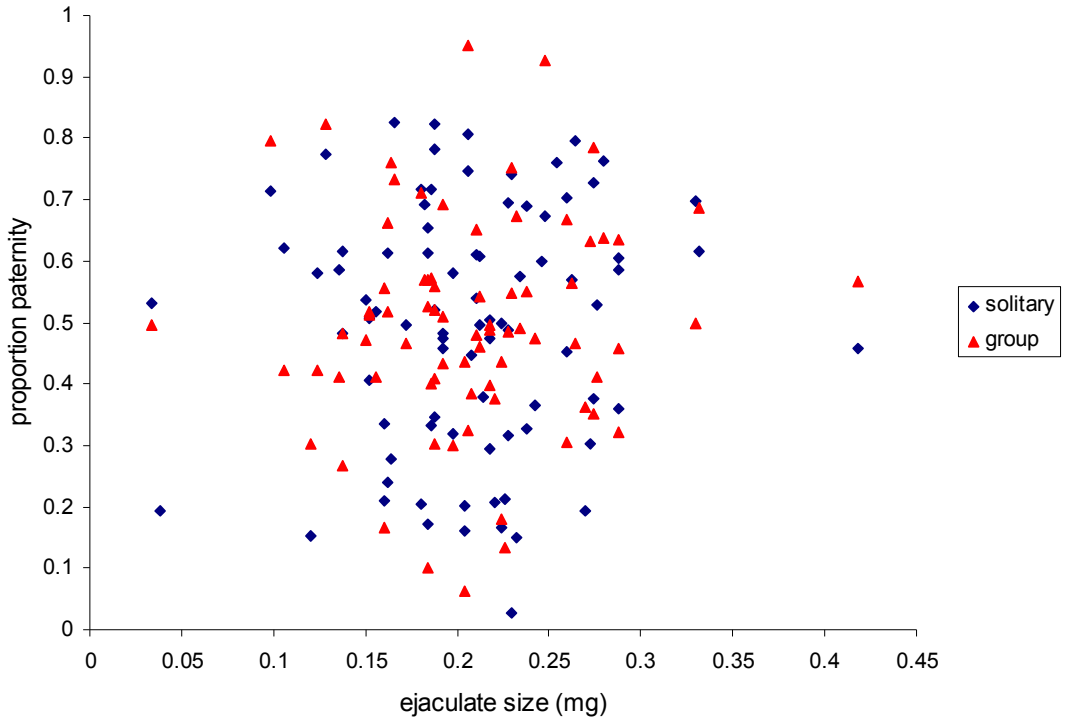

Figure 3.4: ejaculate size and proportion paternity. Ejaculate size is given in milligrams. Male reproductive success is given as the proportion of a clutch of eggs fertilised by a focal male. Blue points represent males from the solitary social context treatment and red points represent males from the group social context treatment. Each data point represents one male. For solitary treatment males, n (sample size) = 84 and for group males, n = 79.

Since mating order affected the proportion of paternity achieved by the focal male, its effect on ejaculate size was also investigated; mating order did not affect ejaculate size ($F_{1, 161}$ = 2.22, p = 0.138); males mating with non-virgin females (second-mating males) did not allocate ejaculates of different sizes than males mating with virgin females (first-mating males).

The effect of social context and ejaculate size on total female fecundity (total number of eggs laid, irrespective of which male they were fertilised by) was investigated; social context did not affect female fecundity ($F_{1, 161} = 0.04$, $p = 0.848$), nor did ejaculate size ($F_{1, 160} = 0.28$, $p = 0.597$); females receiving larger ejaculates from group males did not lay larger numbers of eggs than females receiving smaller ejaculates from solitary males.

For the block in which paternity was analysed using genetic markers, male reproductive success was also analysed as both offspring number and proportion paternity. To analyse offspring number, a general linear model was fitted with social context as the explanatory factor. Social context did not affect the number of offspring males produced ($F_{1, 92} = 0.00$, $p = 0.945$); see Figure 3.5.

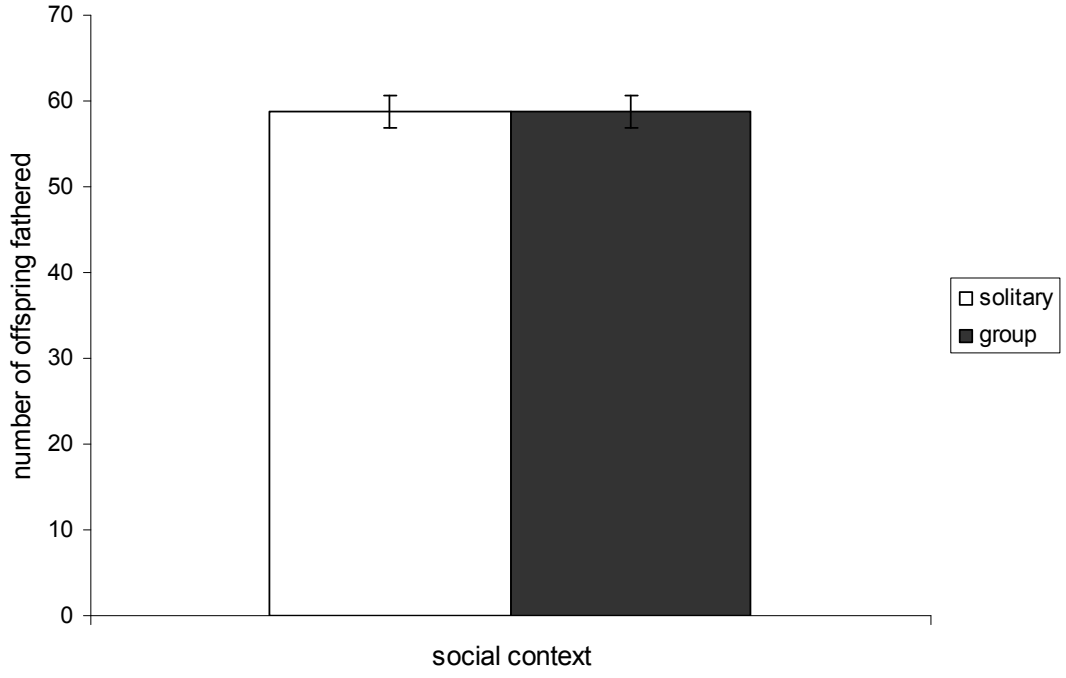

Figure 3.5: social context and offspring number. Male reproductive success is given as the mean number of offspring fathered by males in each social context treatment. The empty bar represents males from the solitary treatment and the filled bar represents males from the group treatment. Error bars give the standard error of the mean. For solitary treatment males, n (sample size) = 47 and for group males, n = 47.

To examine potential associations between ejaculate size and paternity within social context treatments, the analysis was repeated but ejaculate size was added as a covariate. Ejaculate size did not affect male reproductive success ($F_{1, 91} = 1.79$, $p = 0.185$), and with it controlled for there was still no effect of social context ($F_{1, 91} = 0.01$, $p = 0.927$).

To measure the reproductive success of males relative to their genetic marker competitors, the proportion of the offspring clutch they fathered was analysed using a generalised linear model in R, with social context as the explanatory factor. Social context did not affect proportion paternity (chi-sq = 1.52, df = 1, p = 0.597). To control for potential effects of ejaculate size on proportion paternity the analysis was repeated but ejaculate size was added as a covariate. Ejaculate size itself did affect proportion paternity (chi-sq = 49.84, df = 1, p = 0.002) - within treatments, males inseminating larger ejaculates tended to achieve greater paternity proportions; see Figure 3.6, but controlling for it did not reveal any effect of social context (chi-sq = 5.27, df = 1, p = 0.303); see Figure 3.7. There was no effect of the interaction between social context and ejaculate size on proportion paternity (chi-sq = 2.79, df = 1, p = 0.454); the effect of ejaculate size on proportion paternity was no different in the two social context treatments.

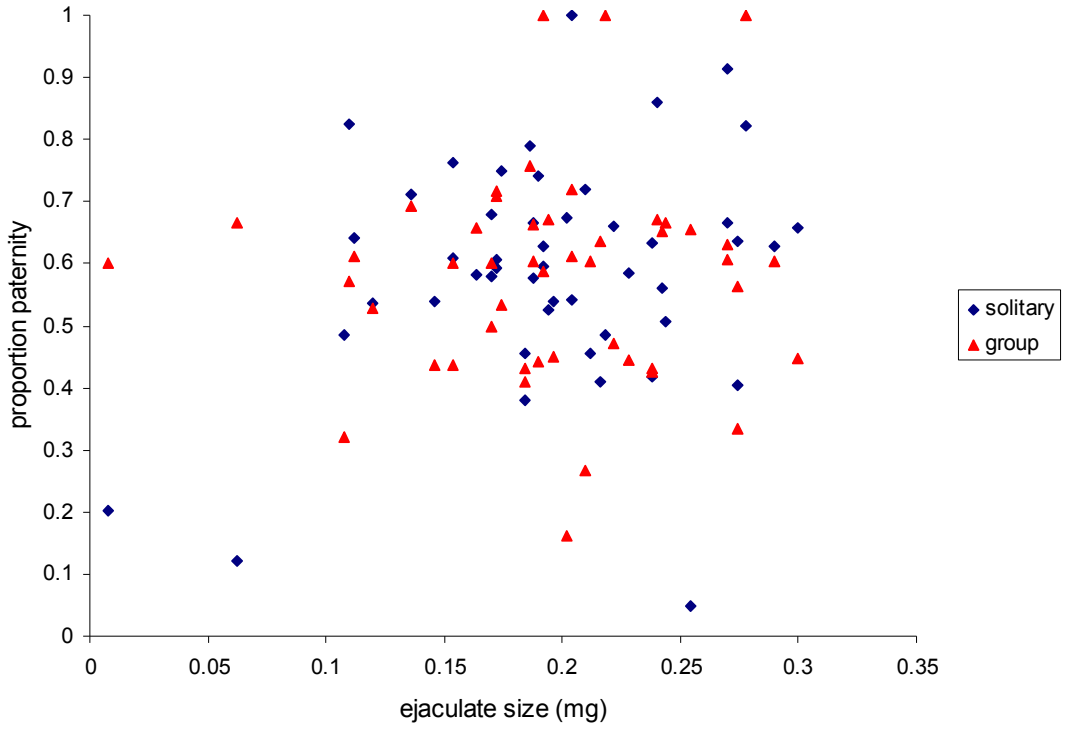

Figure 3.6: ejaculate size and proportion paternity. Ejaculate size is given in milligrams. Male reproductive success is given as the proportion of a clutch of offspring fathered by a focal male. Blue points represent males from the solitary social context treatment and red points represent males from the group social context treatment. Each data point represents one male. For solitary treatment males, n (sample size) = 47 and for group males, n = 47.

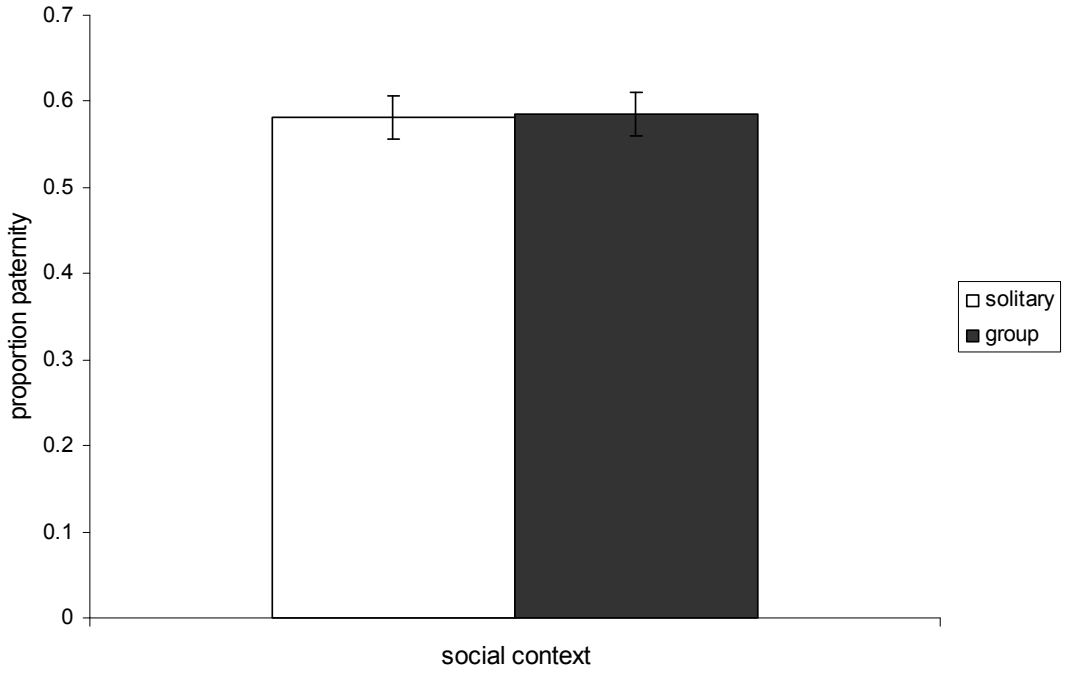

Figure 3.7: social context and proportion paternity. Male reproductive success is shown as the proportion paternity of a clutch, and is given as the mean of males from each social context treatment. The empty bar represents males from the solitary treatment and the filled bar represents males from the group treatment. Error bars show the standard error of the mean. For solitary treatment males, n (sample size) = 47 and for group males, n = 47.

3.3.3. Social context and female receptivity

The propensity of a female to re-mate following copulation with a focal male from the different social context treatments was estimated by analysing the point at which offspring fathered by a second competitor male appeared, following the initial mating with the focal male. Competitor offspring appearance was analysed using a general linear model in Minitab, with social context as the explanatory factor, and the time-point during which eggs fathered by the second competitor male were laid, as the response. Competitor offspring appearance time-point was not affected by whether a female's first mate had experienced solitary or group social context treatment ($F_{1, 97} = 0.72$, $p = 0.397$); see Figure 3.8.

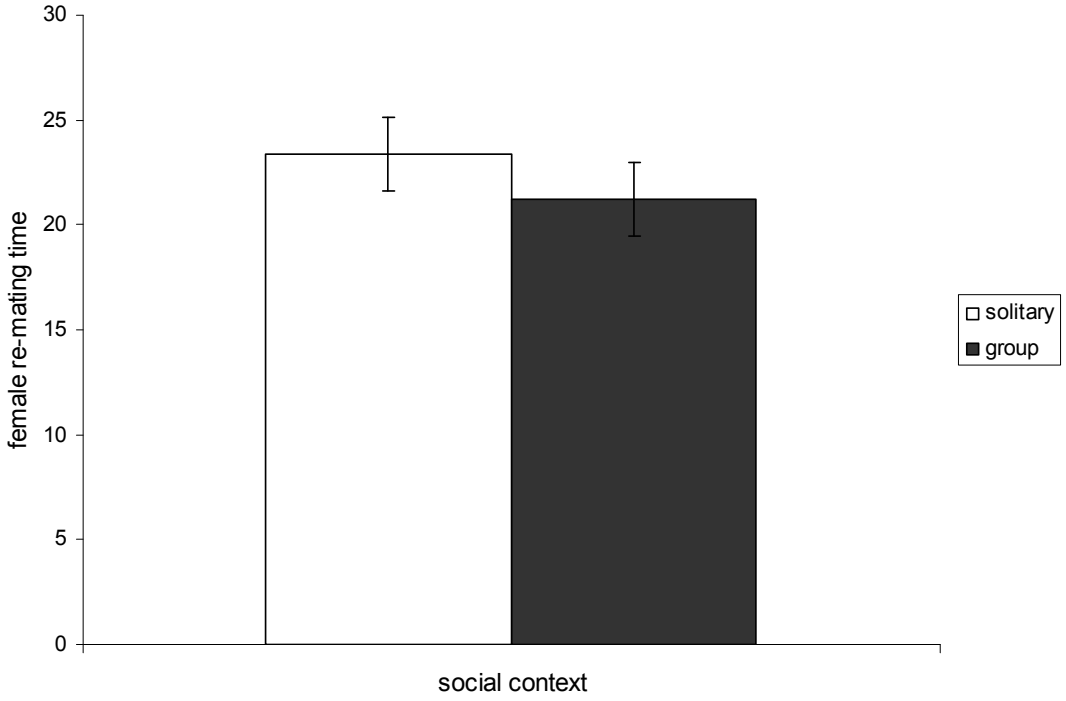

Figure 3.8: social context and female re-mating. Female re-mating is given as the time following initial mating during which females laid eggs fertilised by the second-mating (competitor) males, and is given as the mean for males from each social context treatment. The empty bar represents males from the solitary treatment and the filled bar represents males from the group treatment. Error bars give the standard error of the mean. For solitary treatment males, n (sample size) = 47 and for group males, n = 47.

3.4. Discussion

Males responded to the social context they experienced immediately before mating, by producing larger ejaculates when kept in a group situation than when kept alone. Since there is evidence that increased ejaculate size can both delay female re-mating and increase success in sperm competition in *C. maculatus* (Eady 1994; 1995), this change is predicted by sperm competition theory (Parker 1970), if the presence of other males provides an indication of the risk that a female will mate with rivals. However, in the experiments reported here, this change in ejaculate size caused by social context was not associated with a consequent change in male reproductive success. This result was robust, whether determined by the sterile male

technique or genetic markers, raising questions about whether this behavioural plasticity is adaptive, and whether it has any consequences for post-copulatory sexual selection. My findings do give some evidence for a relationship between ejaculate size and male reproductive success *within* social context treatments; within treatments, ejaculate size did affect proportion paternity, but the effect on ejaculate size of social context treatment was not associated with changes in male reproductive success. This might suggest variation in ejaculate size within social context treatments is due to different ejaculatory components than variation between males in different treatments. In general, therefore, using ejaculate size as a proxy for male reproductive success in insects might not always be suitable; my results suggest a direct measurement of male reproductive success is necessary.

There are reasons to expect that producing a larger ejaculate in response to social context should increase the reproductive success of male *C. maculatus*. In *C. maculatus* last male precedence exists (Eady 1994); the last male to mate with a female before she lays eggs will father most of the offspring in the clutch. It could therefore be advantageous for males to inseminate larger ejaculates to more effectively displace previously-inseminated sperm, and consequently achieve greater fertilisation precedence. I observed first-male precedence in my experiments, because females had 48 hours between matings, during which time they laid large numbers of eggs, which could necessarily only be fertilised by the first-mating male. When only eggs laid after both matings were counted, second-mating males in my experiments did have precedence, corresponding with previous findings (Eady 1994). Whether I measured precedence using the complete clutch, or only those eggs laid after both matings, the degree of precedence was not affected by the social context experienced by males. Second-mating males that had experienced competitor presence, and resultantly produced larger ejaculates, did not achieve greater precedence than second-mating males that remained solitary prior to mating and inseminated smaller ejaculates. Although when measuring male reproductive success as proportion paternity there was a relationship between ejaculate size and male reproductive success within social context treatments, the change in

ejaculate size caused by treatment was not associated with a corresponding change in male reproductive success. In addition, when measuring male reproductive success as the number of offspring fathered, there was no relationship between ejaculate size and offspring number, neither within nor between social context treatments. This allows me to discount the possibility that the adaptive reason for plasticity of ejaculate size in response to social context is that it functions to displace rival sperm, and hence father a greater proportion of the clutch, when the risk of sperm competition is high. The difference in the relationship between ejaculate size and male reproductive success within treatments when measuring offspring number versus measuring proportion paternity might be due to the variability in clutch size; no relationship was found between ejaculate size and the number of offspring fathered, perhaps because this number was so variable, whereas there was evidence of a relationship between ejaculate size and proportion paternity within treatments, perhaps because analysing proportion effectively controlled for clutch size.

Previous studies have suggested that female re-mating in *C. maculatus* is delayed by the insemination of large numbers of sperm (Eady 1995). Another possible advantage to males of inseminating a larger ejaculate could therefore be to increase sperm numbers in order to minimise paternity loss, by delaying a female mating subsequently with a rival male. However, when I used genetic markers as a method of paternity assignment (and hence the time to re-mate was un-manipulated by experimental protocol), I found no evidence that female re-mating was delayed by the larger ejaculates of males having experienced group social context, or that these larger ejaculates affected paternity. Again, despite some evidence of a relationship between ejaculate size and male reproductive success *within* social context treatments, the change in ejaculate size caused by social context *between* treatments did not affect male reproductive success. I therefore discount the hypothesis that the adaptive reason for plasticity of ejaculate size in response to social context is that, under a high risk of sperm competition, a larger ejaculate increases the period of female non-receptivity. From my results, it seems that there is no demonstrable advantage to males of inseminating larger

ejaculates in response to social context, whether they mate before or after a rival male. There is some evidence from other studies that other non-sperm ejaculatory components might delay female re-mating in *C. maculatus*. Yamane *et al* (2008) found that injection of extracts derived from the male reproductive tract into the female abdomen decreased female receptivity, suggesting that greater investment in such products might also delay re-mating. However, I found no association between ejaculate size and female receptivity in this study, suggesting larger ejaculates contain no more of these extracts than smaller ejaculates in this case.

The absence of a relationship between ejaculate size changes in response to rival presence and both male reproductive success and female re-mating, suggests these larger ejaculates contain neither more sperm nor a larger quantity of accessory substances affecting female behaviour. What is up-regulated in larger ejaculates will require further investigation, although there is evidence that water (Edvardsson 2007) or nutrients (Fox 1993; Fox and Moya-Larańo 2009) could be involved. Eady (1995) found that when sperm numbers inseminated by the second-mating male were experimentally reduced, the degree of second male precedence decreased, suggesting that the number of sperm in an ejaculate is key to achieving fertilisations when mating under competitive circumstances. Eady (1995) did not measure ejaculate size or quantify other non-sperm ejaculatory components, therefore it cannot be determined how ejaculate size varied with the differences in sperm numbers, although it would be assumed that ejaculates containing fewer sperm would be smaller in size. However, my results showed that a smaller volume of ejaculate produced by second-mating males exposed to the solitary treatment did not reduce their fertilisation precedence. This suggests that numbers of sperm in ejaculates of different sizes produced in response to social context are, in my experiment, not different. Eady also found that larger numbers of sperm increased the refractory period of female mates (Eady 1995), again suggesting that the sperm component of ejaculate is important in affecting sperm competition and its outcome. Again, the lack of association between ejaculate size and female refractory period in my results suggests sperm numbers do

not differ between ejaculates of different sizes in my study. Further investigations measuring sperm numbers in ejaculates of different sizes in *C. maculatus* are needed before the relationship between ejaculate size and sperm number can be determined. An important follow-up to my study would be to count sperm in the large ejaculates produced by group males, and in the small ejaculates produced by solitary males, to determine whether the lack of effect on male reproductive success in my study, contrasting with the effects on male precedence and female receptivity in Eady's (1995) study, can be explained by differences in how sperm numbers change in ejaculates of different sizes. Because I had not yet received tuition in sperm counting at the time of this study, I did not carry this out, but it is a potentially fruitful course for future investigations.

Despite a correlation between body size and ejaculate size in *C. maculatus* (Savalli and Fox 1998), Savalli and Fox (1999) found that larger males did not induce longer refractory periods in their female mates than did smaller males, suggesting ejaculate size might not always be associated with inducing female non-receptivity. Although I did not measure male body size, the lack of effect of ejaculate size on female receptivity in my study is consistent with this. In another study, however, Savalli and Fox (1999b) found that virgin males inseminating larger ejaculates did delay re-mating by females to a greater extent than did previously-mated males, which inseminated smaller ejaculates. This contrasts with my results, in which female re-mating was unaffected by ejaculate size. The contradictory results from Savalli and Fox (1999; 1999b) might be explained by the difference between ejaculate size variance among males of different sizes, and the changes in ejaculate size resulting from male mating history. The sizes of a male's ejaculates decline dramatically with subsequent matings; Savalli and Fox (1999b) reported a drop from 0.23 mg at first mating to 0.05 mg at third mating. The size variation in ejaculates between males of different size was reported to range from around 0.20 mg for small males (~3.1 mg body weight) up to around 0.40 mg for larger males (~4.7 mg body weight) (Savalli and Fox 1998). Although this is a significant difference in ejaculate size, it might be that unless ejaculates drop below a certain size threshold (which might occur

as males become sperm-limited with subsequent matings), male and female fertility are not affected because of the excess numbers of sperm normally inseminated (Eady 1995).

The refractory period of female *C. maculatus* does question the relevance of present social context to the sperm competition risk a focal male might face. Their socio-sexual surroundings immediately before mating might not reflect the true risk; by the time a female becomes receptive to re-mating (few females re-mate within 24 hours; personal observation), the number of rivals present might have changed. Yamane and Miyatake (2005) demonstrated that males of the closely related *Callosobruchus chinensis* altered ejaculate allocation depending on larval rearing density rather than adult rival presence, suggesting that social context at different stages of the lifecycle might be more indicative of true sperm competition level. I investigate this in *C. maculatus* in Chapters 4 and 5, which examine the effects of larval density on male ejaculate allocation, sperm numbers and reproductive success.

Given the evidence for ejaculate size effects on male fitness in this species, it is unclear why the variation in ejaculate size generated by my treatments had no effect on male reproductive success. It might be that the smaller ejaculates in my dataset are still large enough to comfortably fertilise all the eggs a female lays, so I see no effect on male or female fertility at this level. The mean ejaculate size of solitary males in my experiment was 0.196 mg; although this is smaller than ejaculates produced by group males (a mean of 0.218 mg), it might still be large enough to father as many offspring, and induce as much female non-receptivity, as those produced by group males. Eady (1995) found that males fathered fewer offspring when sperm numbers decreased to around 8,700 from 56,000; the lack of a similar effect in my study could be due to less dramatic differences in sperm provision among my males. Although the males in the Savalli and Fox (1999) study generally produced larger ejaculates than the males in my study, their minimum ejaculate size was around 0.19 mg; males that produced these ejaculates did not induce a shorter period of female non-receptivity

than larger males producing larger ejaculates. My results are consistent with this, and the findings of both studies suggest ejaculate size did not decline enough in my study to adversely affect the ability of males to induce non-receptivity. It would be interesting to repeat my study, but subject solitary and group males to repeated copulations, to see how the effects on male reproductive success and female receptivity change with increased sperm limitation. It is possible group males are investing in sperm competition in a way that would only become evident over several matings, i.e. if ejaculate size diminishes more slowly with time, lifetime reproductive success might be elevated, even though reproductive success in one mating is not.

There is also a more fundamental question; why do males adjust their ejaculate sizes in response to social context, if it has no effect on their reproductive success? The lack of effect of male ejaculate allocation in response to social context on male fertilisation gains and female re-mating receptivity in my study could suggest that plasticity of ejaculate size might not be an adaptive trait, or might have a function other than to affect immediate reproductive success. A possibility is that, rather than signal sperm competition, the presence of rivals in the group treatment indicates some other factor to males that induces production of larger ejaculates, without increasing sperm numbers. Perhaps if males perceive group treatment as crowding, they allocate more water to their ejaculates to benefit females so they can survive long enough to lay more eggs, or so they are strong enough to resist subsequent matings; the male's own reproductive success would be increased by proxy. However, because I found no effect of increased ejaculate allocation in response to social context on female re-mating or fecundity, this is unlikely. It might be that my females are sufficiently healthy and hydrated to lay large numbers of eggs whatever the size of ejaculate they receive, and perhaps if I repeated the study using more stressful conditions, females would have to trade off fecundity with survival; in this case I might see an effect of the change in ejaculate size in response to social context on male reproductive success or female receptivity. Edvardsson (2007) found that females more readily re-mated when they were denied water; to investigate whether

water provision affects ejaculate size, I go on to examine this in Chapter 6. One other possibility is that male ejaculate size increase could represent an investment in offspring quality rather than quantity. There is evidence in *C. maculatus* that when females lay smaller eggs, offspring emerging from them are smaller in size too (Fox and Savalli 1998). If the size of eggs females lay depends on the size of the ejaculates they receive, males inseminating larger ejaculates might father larger offspring. Since body size relates to reproductive success in *C. maculatus* (Colegrave 1993), males could benefit indirectly through the fitness of their offspring. An important extension to this study could therefore be to measure whether egg size varies with the size of ejaculates females receive.

Another possible explanation for the observed effect of social context on ejaculate size is if increased ejaculate allocation in response to rival male presence represents a life-history trade-off of current reproduction against potential future reproduction. It is possible that a larger ejaculate has no effect on female receptivity, and confers no advantage in post-copulatory sexual selection at the current mating, but instead might increase female survival or lifetime fecundity. Although I found no effect on female fecundity, I did not measure longevity, so this might be where the fitness benefit lies (although the fact I found that females did not achieve greater fecundity, regardless of how long they lived, makes this unlikely). If the presence of rival males prior to mating represents a male-biased sex ratio, males might perceive this as indication of a low likelihood of encountering female mates in the future. It would therefore be adaptive for them to invest heavily in the current mating, as saving resources for unlikely future matings might be wasteful. Conversely, males remaining solitary prior to mating have no indicators of sex ratio, and the first individual they encounter is the female they mate with. In their case, they might perceive a greater likelihood of encountering subsequent female mates in the future, so hold back some of their ejaculatory resources for future copulations. There is evidence in other insects that receiving large ejaculates (or multiple ejaculates) can increase female longevity and fecundity. In the turnip moth, *Agrotis segetum*, females mating with males that inseminate smaller ejaculates (having

mated multiple times previously) achieve reduced fertility (Svensson *et al* 1998). In *C. maculatus*, females living under nutrient-limited conditions achieve increased longevity when they mated multiply (Fox 1993). In addition, females mating once every 48 hours achieve greater fecundity than females that mate only once (Fox 1993), although when females are continuously housed with males and mate multiple times, there is no increase in fecundity above that of females mating once (Fox 1993), possibly due to increased harassment costs. This suggests that receipt of multiple ejaculates can increase female longevity and fecundity in *C. maculatus* females; it is possible the same effect could be seen due to the receipt of one, larger ejaculate. An important extension to my study would be to measure the effects of receipt of ejaculates of different size on female longevity. I found no increase in total female fecundity when females received larger ejaculates. This highlights the importance of measuring total offspring number as well as P_1 or P_2, because doing so takes into account any potential effects on female fecundity.

Previous work using other insects has shown social context and sperm competition level to be important in determining male allocation of ejaculate. However, few of these studies have gone on to measure the consequent gains in male reproductive success resulting from increases in sperm or ejaculate allocation (but see Tomkins and Simmons 2000; Bretman *et al* 2009; Lizé *et al* 2012), therefore assumptions that plastic control of reproductive resources is adaptive may not hold true, and require further investigation. Gage and Baker (1991) found that male mealworm beetles, *Tenebrio molitor*, performed as predicted by sperm competition theory by inseminating more sperm when in the presence of a rival. However, resulting male reproductive success was not measured and my results suggest this behavioural plasticity cannot be assumed to be adaptive. Similarly, Simmons *et al* (2007) found that male crickets, *Teleogryllus oceanicus*, responded as expected by increasing the quality of their ejaculates (more live sperm) when exposed to group social context; but again male reproductive success was not measured so the effects of ejaculate adjustment cannot be ascertained.

One of very few studies that has investigated the effects of social context on ejaculate allocation, and taken it through to measure consequent effects on male reproductive success, was using *Drosophila melanogaster*. Bretman *et al* (2009) found that male *D. melanogaster* exposed to rivals increased ejaculate allocation, and as a result achieved greater reproductive success than those not exposed to rivals (Bretman *et al* 2009). However, these results might be confounded by the fact that groups of rival males were left to compete in the mating arena for females; the male achieving the mating would likely be the strongest of the group, so it is unsurprising these males achieved increased reproductive success. In addition, in another recent *Drosophila* study, Lizé *et al* (2012) demonstrated patterns of ejaculate allocation predicted by sperm competition theory even in species without polyandrous mating systems, and found no effect of ejaculate allocation on female receptivity. This raises important questions about the relevance of ejaculate allocation in the *Drosophila* genus, and, along with the lack of reliable evidence for effects on male reproductive success (Bretman *et al* 2009) and female receptivity (Lizé *et al* 2012), means that determining whether plasticity of ejaculate allocation is an adaptive behaviour requires further proof.

In summary my findings suggest that male *C. maculatus* do use their socio-sexual surroundings to assess the risk of sperm competition, and react to increased sperm competition risk by increasing ejaculate size. However, I did not find evidence that this behavioural plasticity is adaptive in this species, as no increase in male reproductive success resulted from increased ejaculate allocation in response to social context. It could be that my treatments did not represent sperm competition levels but rather some other environmental factor such as level of crowding, but there was still no effect of allocation on male fitness, or that males are trading off current against future reproduction, but again I found no demonstrable effect on female fecundity, so this too seems unlikely. My results highlight the need for further studies to carefully measure the fitness consequences for males of plastic ejaculate allocation, and suggest that it cannot always be assumed that apparently adaptive behaviours actually confer fitness benefits. Rather, anatomical or genetic constraints in this

species might have maintained this behavioural plasticity despite its lack of measurable advantage to fitness.

Chapter 4. The effects of larval density on ejaculate size and male reproductive success in *Callosobruchus maculatus*

4.1. Introduction

Sexual selection theory suggests that in situations where the risk of sperm competition varies, and where males can predict and react to this risk, males should evolve to adjust their ejaculate investment in response to cues about sperm competition risk (Parker 1970). In Chapter 3, I showed that ejaculate adjustment occurred in response to adult social context in *Callosobruchus maculatus*, but that there was no measurable effect of this on male fitness. In many species, conditions experienced in early life can have profound effects on physical and morphological development, and therefore on adult behaviour (e.g. Gage and Cook 1994). If early conditions provide information about potential risks of sperm competition in later life, males should be selected to adjust their investment in reproductive resources accordingly. There exists some evidence that this does occur in a variety of insects, including the moth *Plodia interpunctella* (Gage 1995), the armyworm *Pseudaletia separata* (He and Miyata 1997), the cockroach *Nauphoeta cinerea* (Harris and Moore 2005), and the mantid *Pseudomantis albofimbriata* (Allen *et al* 2011). However, larval conditions might also affect reproductive traits for other reasons. In particular, harsh larval conditions might put developmental restrictions on growth or the uptake of resources, or force individuals to trade off reproductive resource allocation against some other developmental factor.

Despite several studies demonstrating effects of larval rearing conditions on male reproductive traits (Gage and Cook 1994; Gage 1995; He and Miyata 1997; Yamane and Miyatake 2005, 2008; Allen *et al* 2011), the consequences of these differences in sperm or ejaculate allocation on male reproductive success have rarely been examined directly. As a result, the adaptive significance of these effects is uncertain. Studies of the Indian meal moth (*Plodia interpunctella*) have shown that manipulation of larval rearing density can affect a

variety of male traits including sperm number (Gage 1995; Gage and Cook 1994), testes size (Gage 1995), larval development time (Gage 1995) and longevity (Gage 1995). Males reared at high densities took longer to develop, emerged with larger testes, and produced more sperm than those reared at low densities (Gage 1995). Males perceive high larval density as indicative of a high risk of sperm competition, and invest more in sexual traits as a result. Similar results might be expected in *C. maculatus* because, like *P. interpunctella*, individuals acquire all their resources during larval growth, have relatively short adult life-spans, and mate several times during their lives. Effects of larval conditions have similarly been observed in other species, with false garden mantid (*Pseudomantis albofimbriata*) males reared in male-biased conditions producing more sperm that those reared in female-biased conditions (Allen *et al* 2011), and in the cockroach, *Nauphoeta cinerea*, males housed with other individuals during the sexual maturation period produce larger spermatophores than those housed alone (Harris and Moore 2005). In the armyworm (*Pseudaletia separata*), males reared at high densities emerge at smaller body size, but produce more apyrene sperm than those reared at low densities (He and Miyata 1997), although eupyrene sperm numbers are unaffected. However, in all of these studies, the adaptive value of these changes in ejaculate were not tested directly, and in some cases the probable effects of demonstrated changes in ejaculate size on male fitness were assumed, based on effects shown in other studies. Few studies have directly measured both ejaculate size changes and male fitness changes as a result of larval density manipulations.

Callosobruchus maculatus is a stored product pest, and there are aspects of its ecology that might favour the evolution of adaptive adjustment in relation to larval density. Because of the nature of rapid rises and falls in population size in the closed habitat of a grain store, the number of other larvae sharing the same bean might give an individual a reliable indication of the likely population density it might face as an adult. When grain stores are densely populated, larvae are likely to develop alongside many others in beans, whereas when a grain store is at the beginning of an infestation, females are likely to disperse eggs more widely

among beans (Mitchell 1975), so larvae are likely to develop solitarily. In the closed environment of a bean store, therefore, and when all individuals are of the normal rather than the dispersal morph (as they are in my lab population), larval density is likely to reliably reflect adult population size. This might in turn indicate the potential level of post-copulatory sexual selection likely to exist; males might therefore be selected to detect and react to larval density by altering ejaculate allocation, in attempt to maximise lifetime reproductive success. If high larval density indicates a high level of sperm competition, males might be expected to react by producing larger ejaculates as adults. There is some evidence that this is indeed the case in a closely related species, the adzuki bean beetle, *C. chinensis*. Yamane and Miyatake (2005; 2008) found that male *C. chinensis* reared at high larval densities produced greater numbers of sperm than those reared at low larval densities, when they belonged to strains with polyandrous mating systems. In contrast, they found that in less polyandrous strains, males reared under high larval densities inseminated fewer sperm than those reared under low larval densities (Yamane and Miyatake 2005), and in strains with intermediate levels of polyandry, sperm numbers were not affected by larval density (Yamane and Miyatake 2008); these results are as expected if sperm number adjustment is an adaptation to sperm competition, since only those males subject to post-copulatory sexual selection should have evolved the behaviour. Males of all strains reared at low larval densities emerged with larger bodies than those reared at high larval densities (Yamane and Miyatake 2005; 2008). The authors conclude that strain-specific levels of polyandry explain their results – the higher re-mating rates of females lead to greater sperm competition for males of polyandrous strains; these males react to this sperm competition, as indicated to them by high larval density, by increasing their sperm allocation (Yamane and Miyatake 2005). Larval density affects the ability of males to react to sperm competition over and above its effect on body size; despite emerging smaller, males from polyandrous strains reared at high larval densities inseminated more sperm than those reared at low larval densities (Yamane and Miyatake 2008). The effects on body size suggest resources are limited when a bean is shared between multiple

larvae; however, despite these limitations, males remain able to inseminate significantly more sperm than males with unlimited resources.

Larval rearing conditions have been previously shown to affect aspects of female fitness in *C. maculatus* (Colegrave 1993; Fox and Savalli 1998); females reared with resource competition emerge as smaller adults, and consequently achieve lower lifetime fecundity (Colegrave 1993), and also lay smaller eggs that yield smaller offspring, than females reared without larval resource competition (Fox and Savalli 1998). It is plausible, then, that larval conditions might affect aspects of male fitness too. There is evidence that larval density does not affect other characters of sperm in *C. maculatus*; sperm length was not affected by larval density (Gay *et al* 2009), although it was affected by maternal age. However, because larval density affects body size at emergence in both females (Colegrave 1993) and males (Gay *et al* 2009), this suggests competition for resources during early life might have important effects on adult life-history, and could therefore affect male reproductive effort and, consequently, success.

In light of the results from Chapter 3, in which plasticity of reproductive resource allocation was demonstrated, but no effects on male fitness were found, I now investigate whether any effects of larval density on ejaculate allocation do lead to the predicted differences in male reproductive success. This could shed light on the relative importance of larval versus adult experience in determining male behaviour in *C. maculatus*, and could indicate what it is that constrains males; resource acquisition or post-copulatory sexual selection. In this chapter, I examine whether larval conditions affect ejaculate size in *C. maculatus*, and test the consequences of this for male reproductive success. Specifically, I test whether high larval density leads males to increase their investment in ejaculate, or whether the reduction in available resources leads to reduced ejaculate size. In contrast to most studies mentioned in other species that look at the effects of larval density on sperm number, I am instead investigating ejaculate size, in order to measure effects on all components of mating effort including non-sperm ejaculatory components. I test whether ejaculate allocation patterns

resulting from different larval rearing densities conform to predictions of either sperm competition risk, or larval crowding limitations. I then go on to measure whether differences in ejaculate allocation caused by larval rearing densities have the expected effects on male reproductive success. If males perceive high larval density as an indication of sperm competition, I predict larger ejaculates from males reared in groups, whereas if males are limited by larval crowding, I predict larger ejaculates from males reared alone in beans.

To manipulate larval density, I rear larvae either solitarily or in groups. I use ejaculate weight as a measure of reproductive effort and then use paternity assignment to assess whether alterations in ejaculate allocation lead to the expected consequent effects on fitness.

4.2. Methods

To investigate the effect of larval density on male ejaculate allocation and male reproductive success, male larvae were reared under either low or high larval density, in either black-eyed beans (large resource) or mung beans (small resource); as adults their ejaculate size was measured and their reproductive success, when mating under competitive circumstances, was calculated.

4.2.1. Manipulating larval density

Approximately 50 non-virgin adult male and female *Callosobruchus maculatus* of the Niamey strain were given around 200 black-eyed beans (*Vigna unguiculata*), and another 50 adults were given around 200 mung beans (*Vigna radiata*). Two different bean types were used so that it could be investigated whether larval density had different effects in beans of different size; mung beans are much smaller than black-eyed beans. In each case, the beetles were left for approximately four hours to lay eggs on the beans, after which time the box was anaesthetised using carbon dioxide gas, and adults were removed. Beans were examined

under a dissecting microscope and numbers of eggs per bean were counted. In black-eyed beans, those with five or fewer eggs were discarded, while those with six or more eggs were kept, and were randomly assigned to one of two treatments – low or high larval density. Beans with fewer than six eggs on were not included in attempt to standardise bean quality – females might lay fewer eggs on poor quality beans (Mitchell 1975). A scalpel was used to scrape off excess eggs from beans to experimentally create the appropriate number of eggs for each treatment; those beans assigned to the low larval density treatment were scraped down to only one egg per bean, and those assigned to the high larval density treatment were scraped to leave five eggs per bean. By only using beans with initially six or more eggs on, all beans included were subject to scraping, therefore standardising conditions. In mung beans, those with three or fewer eggs were discarded, and those with four or more eggs were randomly assigned to either the low or the high larval density treatment. These mung beans were scraped to leave three eggs (in the high larval density treatment), or only one egg (in the low larval density treatment).

Approximately 100 beans in each treatment for both bean types were scraped. All scraping was carried out within six hours of egg laying because within a few days, eggs hatch and larvae enter beans (Howe and Currie 1964); by manipulating egg number early on, the number of larvae entering the bean could therefore be reliably controlled. This process was repeated on four consecutive days (four experimental blocks), so that laying time, and therefore offspring emergence time, was staggered over a number of days, to make the experiment more manageable.

These beans were kept at around 30 °C for three weeks, to allow larval development. After this time, when adult emergence was imminent, all beans were individually isolated (to prevent beetles mating on emergence) in 1.5 ml Eppendorf tubes with ventilation holes. Although only single adults could possibly emerge from the low larval density treatment beans, these beans were also individually isolated, to standardise larval experience. On

emergence, individuals were sexed – males were given individual identification numbers (females were not used). In the case where multiple adults emerged on the same day from the same bean, these individuals were not included in the study; if of opposing sexes they would most likely be non-virgin, and even when multiple males emerged simultaneously, adult exposure to rival males could have altered their ejaculate allocation tactics, therefore potentially confounding results.

Males were weighed to the nearest 0.001 mg on day of emergence, to investigate the effect of larval rearing density on body size, and were left for 48 hours to allow their sperm stores to fully mature before mating (Savalli and Fox 1999). Virgin females were collected from Niamey stock population and were randomly allocated to males from either low or high larval density treatments; virgin females were mated within one day of emergence. Females were anaesthetised using carbon dioxide gas and were weighed to the nearest 0.001 mg both before and after mating. Matings were carried out individually in 55 mm Petri dishes. The weight gain of females during mating was calculated by subtracting the weight before from the weight after mating, and was taken to be an approximation of male ejaculate size (Savalli and Fox 1999). Any females gaining no weight (or losing weight) during mating were considered to have not mated and so were excluded from the study.

4.2.2. Paternity assignment

Genetic markers were used as the method of paternity assignment. In Chapter 3, focal males were of the brown Campinas strain, and black Niamey strain males were used as the competitor genetic markers. In this experiment, however, focal males were of the Niamey strain and competitor marker males were of the Campinas strain, in attempt to make the offspring paternity assignment process easier. In Chapter 3, offspring from the first-mating male were brown, and a second mating with the competitor male was indicated by the appearance of black markings on sections of the bodies of the offspring. By using black focal

males and black females this time, it was hoped offspring fathered by the second brown male would be more easily identified by having a more obvious brown appearance.

Competitor males were acquired as virgins from the Campinas strain culture; these males carry a marker gene giving brown body colouration (in contrast to the Niamey males, which are black). Following copulation with the initial focal males, weighed females were each placed individually in 90 mm Petri dishes with approximately 100 black-eyed beans, and a randomly allocated competitor male was added. These pairs were left at around 30 °C, and were allowed to mate if and when the natural period of female latency expired. By not controlling the time-point of the second mating, ejaculates from first-mating males could influence both sperm competition and female re-mating time. Following their deaths, the females and competitor males were removed, and beans containing larvae were left at 30 °C. After around three to four weeks, offspring emerged from these beans; empty beans were removed and the offspring were left in their dishes until they died (approximately 10 days later). These offspring were then observed under a dissecting microscope and assigned as either black or brown in body colour, using the method described in Chapters 2 and 3. Offspring that were black had been fathered by the first, black (focal) male from either a low or high larval rearing density treatment, while offspring that were brown had been fathered by the second competitor male. The numbers of black and brown offspring from each female were counted, so that the reproductive success of each focal male could be calculated.

The ejaculate sizes of males having been reared in low and high larval density treatments were compared, to investigate the effect of larval density on male ejaculate allocation. The reproductive success of focal males from low and high larval density treatments when mating under competitive circumstances were compared, to investigate the effect of larval density on adult male reproductive success.

4.2.3. Statistical analyses

Analyses of the effects of larval density on male body size and ejaculate size were carried out using Minitab 15, and analyses of the effects on male reproductive success were done using both Minitab and R. When General Linear Models were carried out in Minitab, all explanatory factors and covariates were initially included, along with their interactions, to produce the maximal model; non-significant interactions were then removed one by one until the minimal model was obtained. The minimal model included all factors that related to the experimental design (e.g. larval density treatment, bean type and block), whether or not they had significant effects on the response, and any interactions that did affect the response. All stated statistics are from minimal models. For Minitab paternity analyses, offspring number was square-root transformed, to fit the assumption of a normal distribution. The reproductive success analyses in R were Generalised Linear Models with quasibinomial errors; between-beetle variation meant that the residual deviance in binomial models was too high, so quasibinomial models were used. Models in R were initially fitted with all factors, covariates and their interactions. Non-significant interactions were sequentially removed, and the minimal models were those inclusive of relevant experimental design factors (larval density treatment, bean type and block); in different models male body size and/or ejaculate size were also included as covariates – details of the models are given in relevant sections below. The significance of terms were taken from ANOVAs comparing models with and without the terms of interest.

Although all interactions were tested, analyses revealed no interactions were significant; for simplicity, results of interactions are only presented if they are relevant to the questions being addressed.

4.3. Results

4.3.1. Larval density and male body size

Male body size at emergence was not affected by larval density ($F_{1, 111} = 1.86$, $p = 0.176$); see Figure 4.1. Male size was also unaffected by bean type ($F_{1, 111} = 0.67$, $p = 0.414$) and block ($F_{3, 111} = 1.01$, $p = 0.391$).

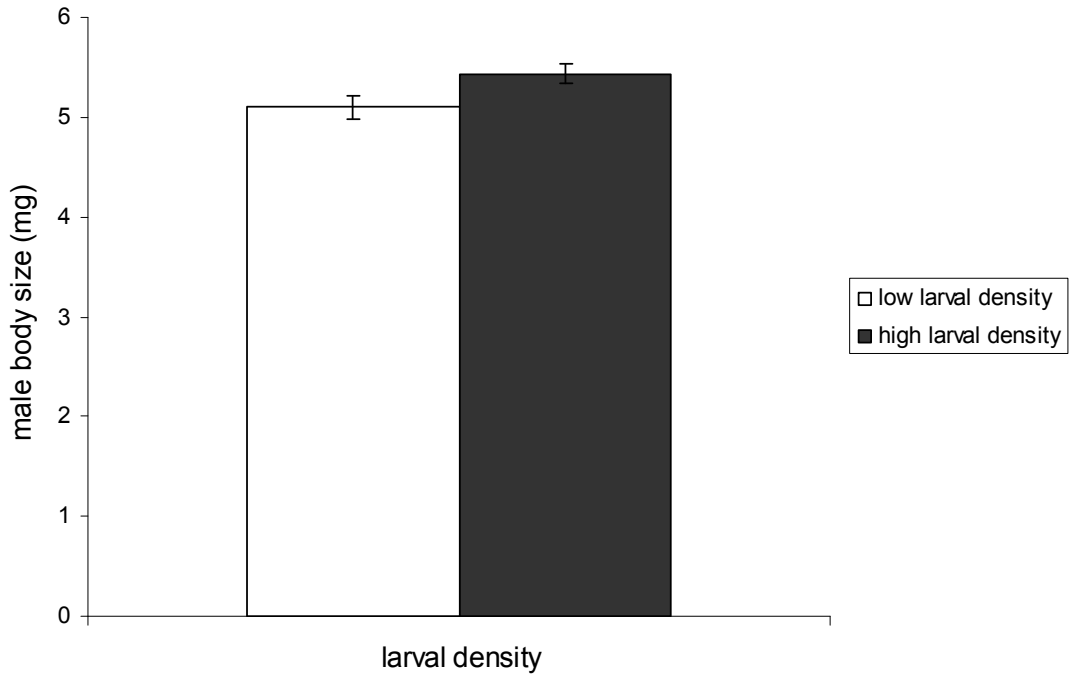

Figure 4.1: larval density and male body size. Male body size given is the mean value in milligrams of each larval density treatment group. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars show the standard error of the mean. For low larval density males, n (sample size) = 51 and for high larval density males, n = 66.

4.3.2. Larval density and ejaculate size

Larval density did not affect ejaculate size ($F_{1, 106} = 1.53$, $p = 0.219$), nor was there any effect of bean type ($F_{1, 106} = 0.29$, $p = 0.589$). Block did affect ejaculate size ($F_{1, 106} = 3.05$, $p = 0.032$) but there was no effect of the interaction between larval density and block ($F_{3, 102} = 1.36$, $p = 0.259$) - effects of larval density on ejaculate size were inconsistent across blocks; see Figure 4.2.

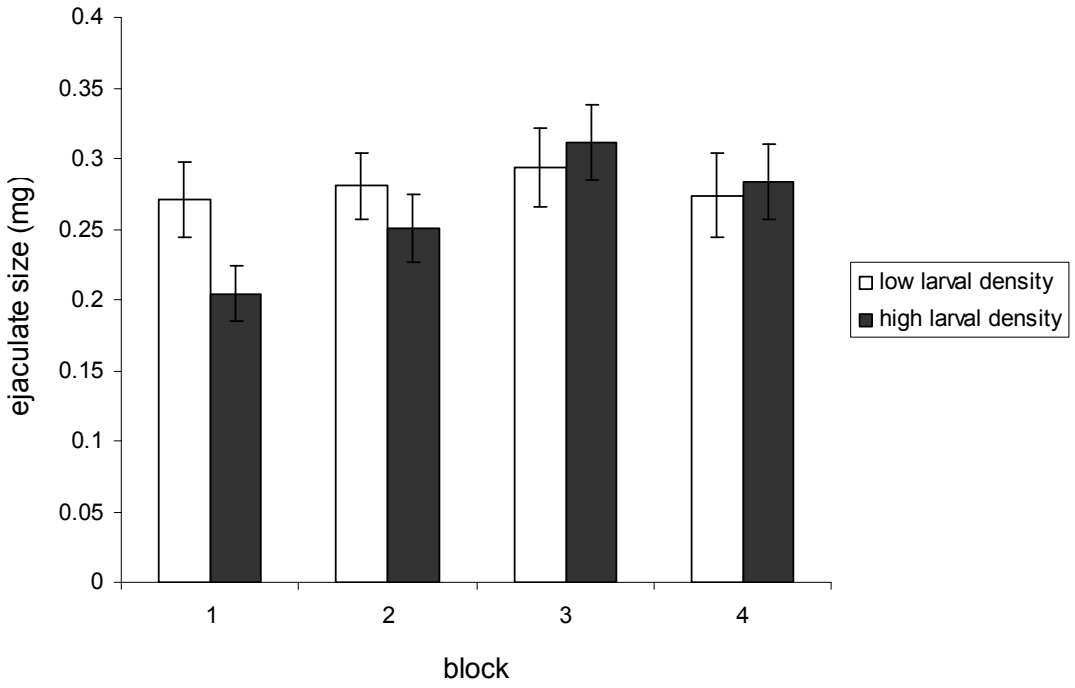

Figure 4.2: larval density, ejaculate size and block. Ejaculate size given is the mean value in milligrams of each larval density treatment group, within each of four experimental blocks. Empty bars represent males from the low larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. For block 1 low larval density males, n (sample size) = 12 and for high larval density males, n = 23; for block 2 low larval density males, n = 16 and for high density males, n = 15; for block 3 low larval density males, n = 11 and for high larval density males, n = 12; and for block 4 low larval density males, n = 10 and for high larval density males, n = 13.

Ejaculate size is known to be affected by male body size in this species (Savalli and Fox 1999). To control for the effect of variation in male body size on ejaculates, the analysis was repeated but with male body size included as a covariate. Ejaculate size in this study was indeed positively related to male body size ($F_{1, 105} = 21.53$, $p < 0.001$); see Figure 4.3, and once male body size was controlled for, there was a borderline effect of larval density on ejaculate size ($F_{1, 105} = 3.74$, $p = 0.056$) - males from high larval densities tended to produce smaller ejaculates for their size than males from low larval densities; on average ejaculates produced by males from low larval densities were around 10.95 % larger than those produced by males from high larval densities, for their body sizes. There was no effect on ejaculate size of the interaction between larval density and bean type ($F_{1, 98} = 0.33$, $p = 0.569$); see Figure 4.4.

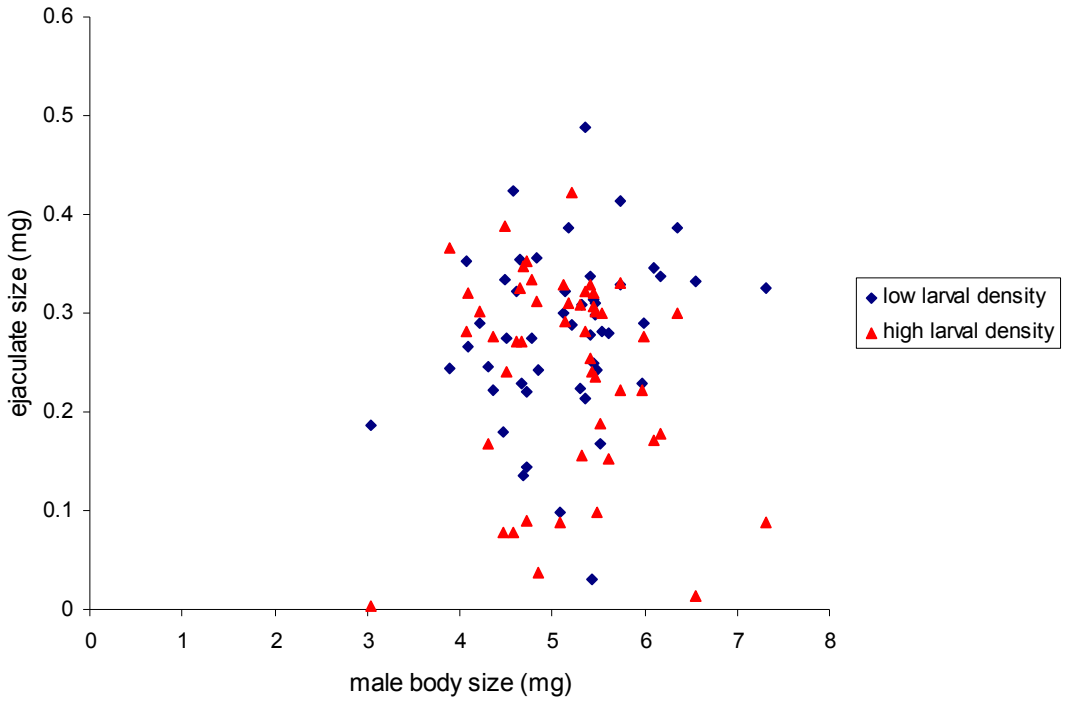

Figure 4.3: male body size and ejaculate size. Ejaculate sizes and male body sizes are given in milligrams. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 49 and for high larval density males, n = 64.

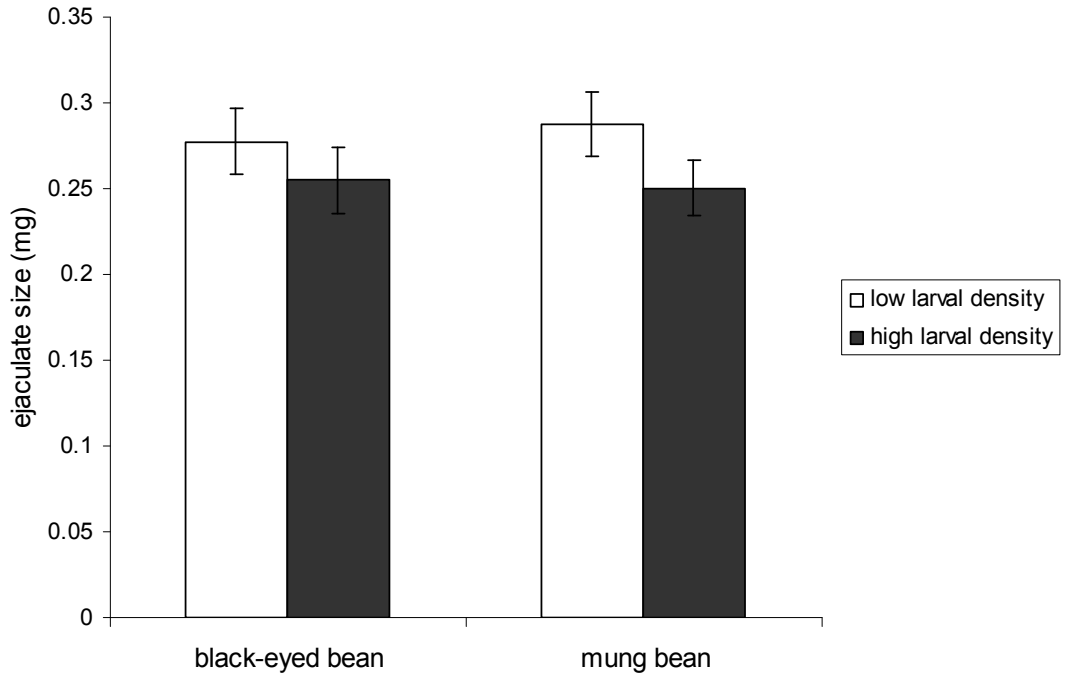

Figure 4.4: larval density, bean type ejaculate size. Ejaculate size given is the mean value in milligrams of each larval density treatment group, in each bean type. Empty bars represent males from the low larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. From black-eyed beans, low larval density male n (sample size) = 24 and high larval density male n = 31, and from mung beans, low larval density male n = 25 and high larval density male n = 32.

4.3.3. Larval density and male reproductive success

Two measurements of male reproductive success were analysed – the number of offspring fathered, and the proportion of the entire clutch fathered, by focal males. By analysing offspring number, I test whether a male's response to larval density affects his total fitness, and by analysing the proportion of the clutch fathered, I examine whether his response to

larval density affects his fitness relative to that of another male, when mating under competitive circumstances.

To examine whether there was a general effect of larval density on offspring number, a general linear model was fitted including the factors larval density, bean type and block, and their interactions. Larval density did affect male reproductive success ($F_{1, 106} = 5.16$, $p = 0.025$) - males reared at low larval density fathered about 30.42 % more offspring than those reared at high density; see Figure 4.5. Male reproductive success was unaffected by bean type ($F_{1, 106} = 0.66$, $p = 0.420$) and block ($F_{3, 106} = 0.92$, $p = 0.436$). There was no effect of the interaction between larval density and bean type ($F_{1, 99} = 0.27$, $p = 0.602$).

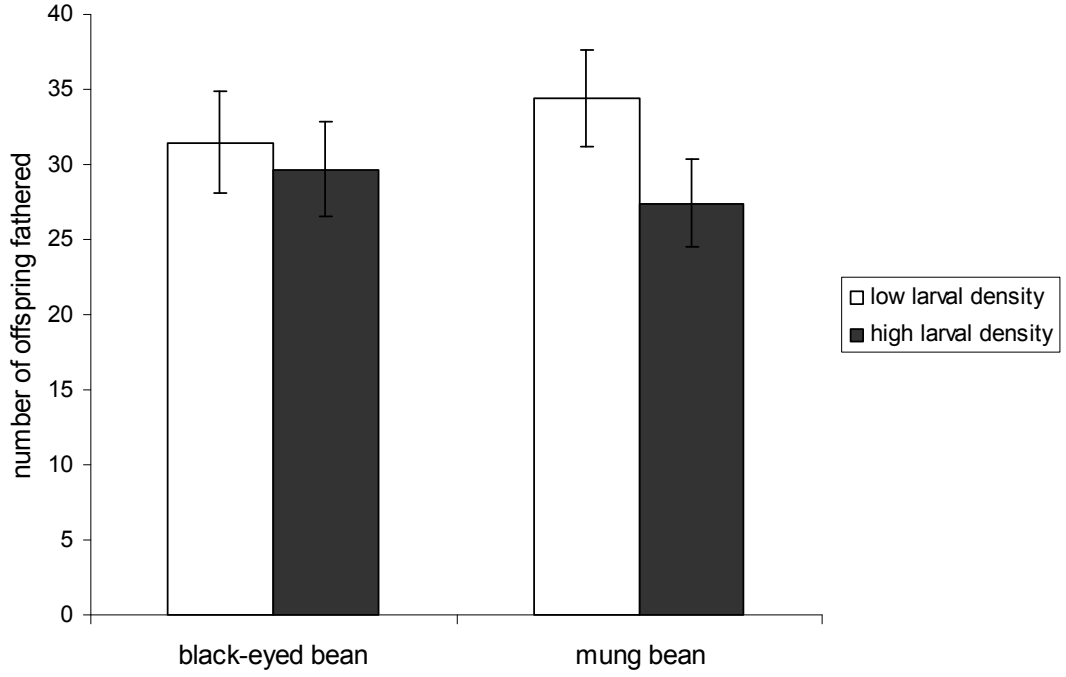

Figure 4.5: larval density, bean type and offspring number. Male reproductive success is given as the mean number of offspring fathered by the focal males having experienced low or high larval densities, in each bean type. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars show the standard error of the mean. From black-eyed beans, low larval density male n (sample size) = 24 and high larval density male n = 31, and from mung beans, low larval density male n = 25 and high larval density male n = 32.

To control for potential effects of male body size on reproductive success, the analysis was repeated but male body size was added to the model as a covariate. Male size did have a borderline affect on male reproductive success ($F_{1, 105} = 3.54$, $p = 0.063$); see Figure 4.6, and once male size was controlled for larval density had a more significant effect on male reproductive success ($F_{1, 105} = 6.36$, $p = 0.013$); males from low larval densities fathered about 33.37 % more offspring for their body size than males from high densities. The effect of larval density on male reproductive success did not differ between bean types – there was no effect of the interaction between larval density and bean type ($F_{1, 98} = 0.10$, $p = 0.749$).

Neither bean type ($F_{1, 105} = 0.89$, $p = 0.347$) nor block ($F_{1, 105} = 0.84$, $p = 0.474$) affected male reproductive success.

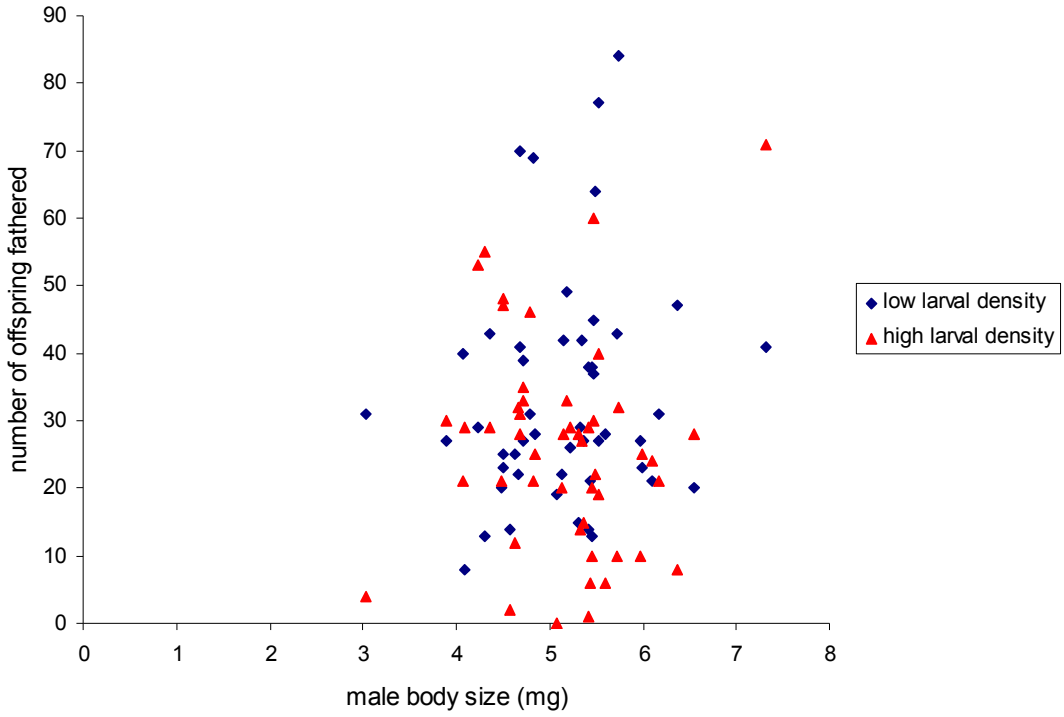

Figure 4.6: male body size and offspring number. Male body sizes are given in milligrams. Male reproductive success given is the number of offspring fathered by focal males. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 49 and for high larval density males, n = 63.

One possible mechanism of the effect of larval density on male reproductive success is via its effect on ejaculate size (see Figure 4.4). To examine this, the analysis was repeated, but ejaculate size was added as a second covariate. With ejaculate size controlled for, larval density affected male reproductive success ($F_{1, 104} = 4.74$, $p = 0.032$); low density males fathered about 25.01 % more offspring for the size of their ejaculate than high density males did. This effect did not differ between bean types (no effect of the interaction between larval density and bean type ($F_{1, 97} = 0.05$, $p = 0.819$). Male reproductive success was unaffected by

bean type ($F_{1, 104} = 1.00$, $p = 0.319$), block ($F_{3, 104} = 0.43$, $p = 0.733$) and male size ($F_{1, 104} = 1.01$, $p = 0.317$), but ejaculate size had a borderline effect ($F_{1, 104} = 3.09$, $p = 0.082$) - males producing larger ejaculates tended to father more offspring; see Figure 4.7.

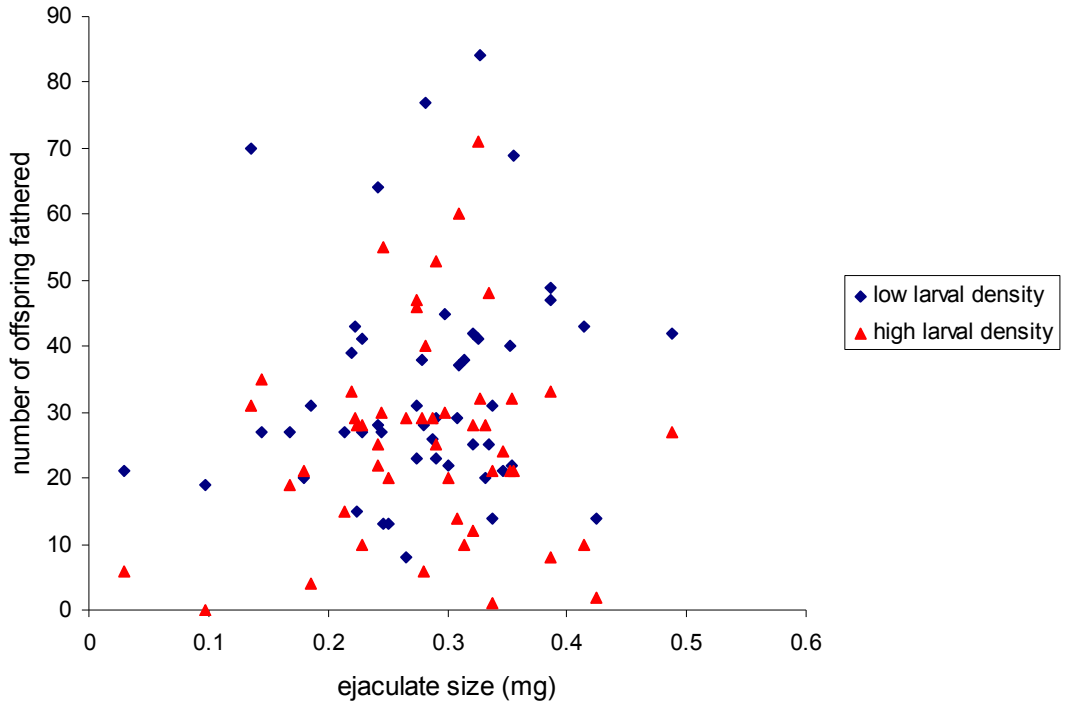

Figure 4.7: ejaculate size and offspring number. Ejaculate sizes are given in milligrams. Male reproductive success given is the number of offspring fathered by focal males. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 49 and for high larval density males, n = 63.

Because larval density still affects male reproductive success when both male body size and ejaculate size are controlled for in the model, this suggests larval density affects male reproductive success over and above its effects via ejaculate size, and, as well as affecting absolute reproductive success, larval density affects success corrected for body size.

To examine whether there was an effect of larval density on the proportion of the clutch fathered by the focal male (relative to that of his competitor), a generalised linear model was fitted in R, with larval density, bean type and block as factors. Neither block (chi-sq = 11.23, df = 3, p = 0.855) nor bean type (chi-sq = 10.54, df = 1, p = 0.396) affected proportion paternity, but there was a borderline effect of larval density (chi-sq = 49.5, df = 1, p = 0.067) - males from high larval densities tended to father a smaller proportion of the clutches than did males from low larval densities; see Figure 4.8. There was no effect of the interaction between larval density and bean type (chi-sq = 5.42, df = 1, p = 0.546).

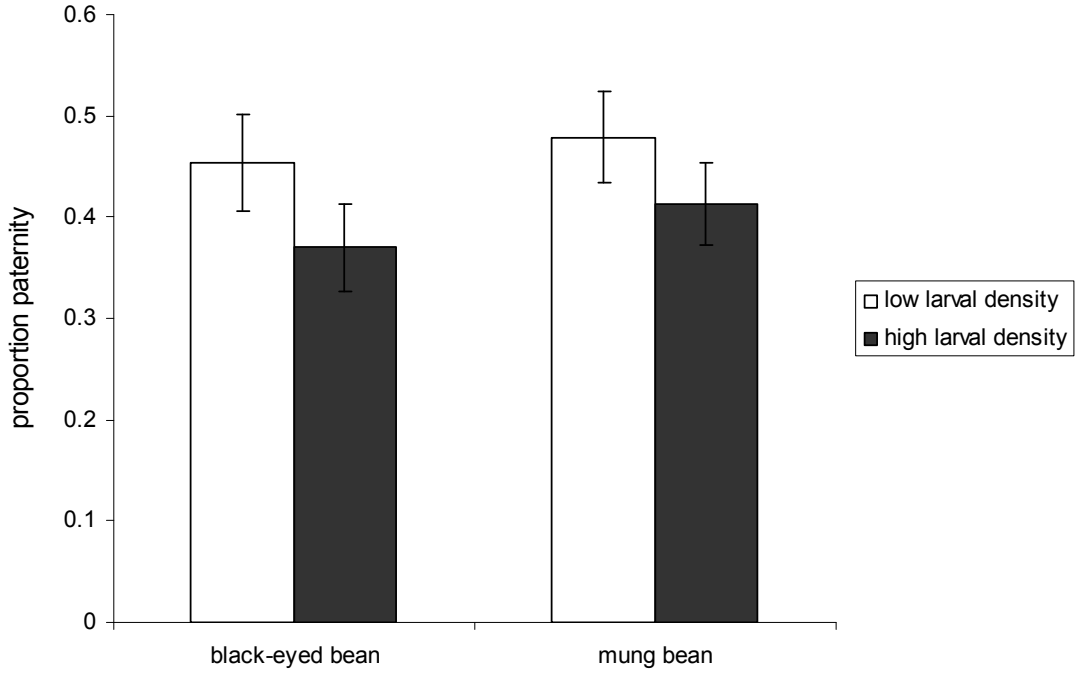

Figure 4.8: larval density, bean type and proportion paternity. Male reproductive success is given as the mean proportion of the total clutch fathered by the focal males having experienced low or high larval densities, in each bean type. Empty bars represent males from the low larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. From black-eyed beans, low larval density male n (sample size) = 24 and high larval density male n = 31, and from mung beans, low larval density male n = 25 and high larval density male n = 32.

To control for potential effects of male body size on reproductive success, the analysis was repeated, but male size was added as a covariate. Male body size itself did not affect proportion paternity (chi-sq = 22.49, df = 1, p = 0.216) but, when it was controlled for, larval density did affect male reproductive success (chi-sq = 57.16, df = 1, p = 0.050), suggesting males reared at low densities achieve greater reproductive success for their body size than do males reared at high larval densities. There was no effect of the interaction between larval density and bean type (chi-sq = 2.14, df = 1, p = 0.703); the effect of larval density on proportion paternity did not differ between beans. Male reproductive success was unaffected by block (chi-sq = 12.81, df = 3, p = 0.830) and bean type (chi-sq = 12.6, df = 1, p = 0.354).

To test whether the effect of larval density on proportion paternity was entirely due to its effect on ejaculate size, the analysis was repeated but ejaculate size was included as a covariate. Ejaculate size itself did not affect proportion paternity (chi-sq = 32.41, df = 1, p = 0.138); see Figure 4.9, and when it was controlled for in the model, the effect of larval density on proportion paternity returned to borderline (chi-sq = 40.23, df = 1, p = 0.099), suggesting the effect of larval density is due to its effect on ejaculate size. There was no effect of the interaction between larval density and bean type on proportion paternity (chi-sq = 0.1.15, df = 1, p = 0.780). Male reproductive success was unaffected by bean type (chi-sq = 13.83, df = 1, p = 0.331), block (chi-sq = 8.44, df = 3, p = 0.900) and male body size (chi-sq = 3.82, df = 1, p = 0.609).

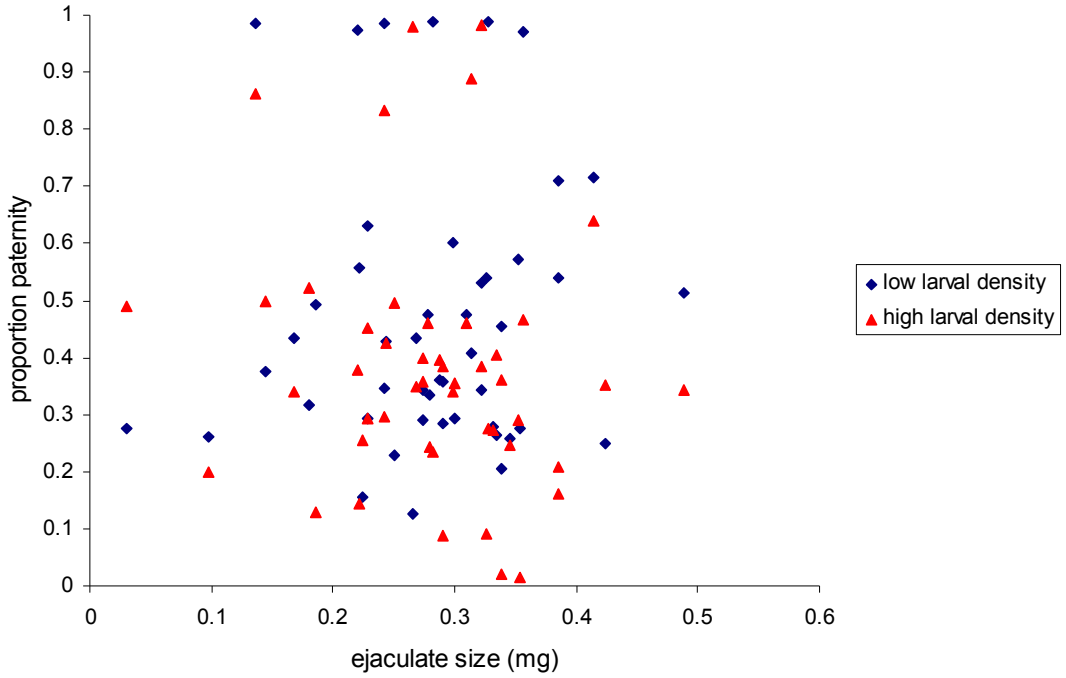

Figure 4.9: ejaculate size and proportion paternity. Ejaculate size is given in milligrams. Male reproductive success is calculated as the proportion of the whole clutch fathered by the focal males, having experienced low or high larval densities. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. From black-eyed beans, low larval density male n (sample size) = 24 and high larval density male n = 31, and from mung beans, low larval density male n = 25 and high larval density male n = 32.

When male reproductive success is measured as offspring number, there are clear effects of larval density over and above effects on ejaculate size, whereas when it is measured as clutch proportion, the effect of larval density is borderline when ejaculate size is included in the model. This suggests the models using clutch proportion in R might have less statistical power than those carried out in Minitab using offspring number.

Finally, to examine whether the different patterns seen for the different measures of reproductive success might be explained by changes in total female fecundity, the effect of male larval density on the total fecundity of their female mates was investigated (with total

lifetime offspring number produced by females as the response). Total female fecundity was not affected by male larval density ($F_{1, 104} = 1.47$, $p = 0.229$), or by ejaculate size ($F_{1, 104} = 0.42$, $p = 0.518$). Females receiving larger ejaculates from males reared at low larval densities did not produce more offspring during their lives (fathered by either of two competing males) than females receiving smaller ejaculates from males reared at high larval densities.

4.4. Discussion

Larval density did affect male reproductive success; males reared at high larval density fathered fewer offspring, and smaller proportions of clutches, than those reared at low larval density. This reflects the similar trend for males reared at high density to produce smaller ejaculates, although the significant effect of larval density on male reproductive success (offspring number) when ejaculate size was controlled for, suggests a high larval density impedes male reproductive success more than just via its effect of reducing ejaculate size. The size of the direct effect of larval density on reproductive success (a 25.01 % difference), compared to the size of its effect via ejaculate size (a 30.42 % difference), suggests the majority of the overall effect of larval density on reproductive success is due to direct effects, rather than effects via ejaculate size. I found a borderline effect of the ejaculate size of first-mating males on offspring number. This contrasts with previous findings in which sperm numbers inseminated by first-mating males did not affect their degree of paternity success, whereas sperm numbers inseminated by second-mating males did (Eady 1995). It is possible that this inconsistency is because I measured ejaculate size, whereas Eady (1995) measured sperm numbers; perhaps non-sperm ejaculatory components are responsible for the increased male reproductive success from larger ejaculates in my study. To begin to investigate this, in Chapter 5 I go on to measure sperm numbers in ejaculates produced by males reared at high and low larval density. Larval density had no effect on male body size; this contrasts with findings in some insect species (Yamane and Miyatake 2005; 2008; He and Miyata 1997), but is consistent with others (Gage 1995; Allen *et al* 2011).

The two measures of male reproductive success show broadly similar patterns. This is not entirely surprising; the fact that larval density did not affect the total clutch size means that offspring number and proportion paternity are in fact really measures of the same thing. This suggests that the difference in statistical significance seen for the independent effects of larval density on reproductive success for the two measures probably does not indicate a difference in biological effect, but is simply due to a difference in power of the two tests used. On balance, the fact that one measure shows a significant effect, and the other shows the same pattern but is marginally non-significant, probably does suggest that there is a direct effect of larval density on paternity, independent of effects on overall ejaculate size. Potential reasons for this effect of larval density on male reproductive success are explored in Chapter 5.

In contrast to Chapter 3, in which differences in ejaculate size due to social context did not affect male reproductive success, the differences in ejaculate size here caused by larval density clearly do affect male fitness. This suggests adult and larval conditions might affect different components of the ejaculate. It is possible that larval conditions strongly influence the numbers of sperm males are able to produce, but once beetles emerge as adults, they are constrained and thereafter can only alter other ejaculatory components. However, the fact that males continue to produce sperm throughout their lifetimes (Eady 1991) makes this unlikely.

Larval density is known to affect the emergence size and fecundity of female *C. maculatus* (Colegrave 1993). This study shows that larval competition can affect male fitness too, but in less obvious ways. Being reared at high larval density is a disadvantage to male *C. maculatus*; they achieve lower reproductive success than those reared at low larval density. This suggests group rearing constrains the quantity of resources males can acquire in a shared bean, whereas males reared in their own bean are able to invest more heavily in reproduction. That males did not emerge smaller from high larval density beans, but did have lower fitness,

suggests they might be trading off body size with reproductive investment, and are prioritising body size. It would be interesting to repeat the study and measure testes size to see whether this is traded off against body growth; larval density has been shown to affect testes size in other insects (Gage 1995). Body size might be more important than sperm number or ejaculate size in *C. maculatus*, because achieving copulation can be difficult, as females tend to be larger than males, they often flee from courting attempts (personal observation), and try to prevent mating by kicking males off (Crudgington and Siva-Jothy 2000). In addition, in the wild when population density is low, finding a mate might be time-consuming. Being larger allows males to move more quickly, therefore females might be more easily located. My results suggest males are selected to grow large bodies; a male instead trading off body size in favour of reproductive resources might have more sperm to inseminate, but might not be able to mate at all if he is not large enough to catch a female. Male body size in *C. maculatus* has been found to affect male mating and reproductive success; larger males more often achieve matings than their smaller competitors (Savalli and Fox 1999). Larger males have also been found to induce increased female lifetime fecundity due to the larger ejaculates they produce (Savalli and Fox 1999). Since male size affects the probability of getting a mate at all, it might be important in *C. maculatus* to maintain body size, even if that means sacrificing some investment in reproduction, and thus losing some paternity if females subsequently go on to re-mate.

My results contrast notably with studies in *C. chinensis* by Yamane and Miyatake (2005; 2008). They found males from polyandrous strains reared at high larval density produced more sperm, despite emerging with smaller bodies. In monandrous and lowly polyandrous strains, they found males from high larval density produced fewer sperm (Yamane and Miyatake 2005; 2008). My population of *C. maculatus* is highly polyandrous, yet my high density males did not emerge smaller, but produced smaller ejaculates and achieved lower reproductive success. Yamane and Miyatake did not measure male reproductive success, but concluded males were reacting to the high levels of sperm competition represented by high

larval density, by increasing their investment in reproductive resources and consequently inseminating more sperm (Yamane and Miyatake 2005; 2008). It seems my population of *C. maculatus* are constrained by resource acquisition at high larval density, whereas their population of *C. chinensis* are reacting to sperm competition, and instead trade off their body size in order to produce more sperm. Yamane and Miyatake suggest that in their monandrous strains, high larval density constrains body size, which in turn constrains sperm number, and that males of these strains are not selected to react to sperm competition because of historically low levels of female re-mating, so do not increase sperm numbers in reaction to high larval density (Yamane and Miyatake 2008). My high larval density males showed reduced ejaculate size (and reduced reproductive success), consistent with their results in the monandrous strains; this is surprising because my population of *C. maculatus* is highly polyandrous, so my males would be expected to be selected to react to sperm competition by increasing ejaculate allocation. The degree of sexual size dimorphism in *C. maculatus* is greater than in *C. chinensis* (Southgate *et al* 1957; Southgate 1958); this might be responsible for the differences between my findings and those of Yamane and Miyatake (2005; 2008). However, that male *C. chinensis* are more similar in body size to females suggests they have been selected to maintain body size because it is an important aspect of fitness. It seems surprising, then, that when reared at high larval density, body size seems to be sacrificed in order to instead elevate sperm number (Yamane and Miyatake 2005; 2008). It is possible that in *C. maculatus*, males are selected to emerge at the minimal body size necessary to be able to successfully copulate, so, although they develop at high larval density, body size is not reduced any further, otherwise mating might be impossible. Instead, sperm numbers or ejaculate investment might be sacrificed due to resource limitation.

Another possible reason for the differences in results in *C. maculatus* and *C. chinensis*, is that my *C. maculatus* males were 48 hours old at the time of mating, whereas the *C. chinensis* males mated at 24 hours of age or younger (Yamane and Miyatake 2008). In *C. maculatus*, it can take 48 hours from emergence for all sperm reserves to be mobilised (Savalli and Fox

1999); if a similar physiological effect occurs in *C. chinensis*, it is possible the results to not reflect maximal sperm numbers, although they are likely to be more representative of natural scenarios, in which newly emerged males mate as soon as the opportunity arises. It would be interesting to repeat both the *C. chinensis* and *C. maculatus* studies, but subject males to several subsequent matings, to determine whether larval density affects the rate at which ejaculatory reserves diminish. Yamane and Miyatake (2005; 2008) held their adult males in groups after emergence, and males from high density conditions were pooled and then randomly collected for inclusion in the study; their results could therefore be confounded by effects of adult social context, which have been shown to affect ejaculate allocation in a number of insects (Schaus and Sakaluk 2001; Gage and Barnard 1996; Engqvist *et al* 2007; Tomkins and Simmons 2000). However, in a separate experiment they found no effect of adult social context on sperm numbers (Yamane and Miyatake 2005).

Sperm numbers and ejaculate size have previously been shown to affect female receptivity in *C. maculatus* (Eady 1995). Although my experimental protocol allowed a natural period of female latency (the time of second matings were not controlled), I did not directly measure female re-mating time. It would be interesting to repeat the study, but to measure female non-receptivity period. In Chapter 3, increased ejaculate size due to adult social surroundings did not influence female receptivity, but because it also had no effect on male reproductive success, I might see an effect on female re-mating this time, if larval and adult conditions influence different aspects of male ejaculate allocation. In Chapter 3, ejaculate sizes ranged from around 0.196 mg to around 0.218 mg (and all males developed solitarily in beans). In the current experiment, ejaculates varied from around 0.250 mg in high larval density males to 0.288 mg in low larval density males; that these ejaculates were generally larger, or that there was a bigger difference between the sizes of ejaculates from males in different treatments, might explain why I see an effect of ejaculate size on male reproductive success this time, but I did not in Chapter 3.

Previous work using other insects has shown larval density to affect male ejaculate allocation (Gage 1995; Gage and Cook 1994; He and Miyata 1997; Allen *et al* 2011). In the Indian meal moth, males reared at high larval density produced more sperm, took longer to develop and had larger testes and abdomens than those reared at low density (Gage 1995). Like *C. chinensis*, *P. interpunctella* appear to perceive high larval density as indicative of sperm competition, and react by investing highly in sexual traits; males reared at low density invest more in longevity and in mate-searching, by growing larger heads and thoraces (which include flight structures), as a result of the perception of low population density (Gage 1995). It is interesting that, like my *C. maculatus* males, these males emerge with the same overall body sizes irrespective of larval density – it would be interesting to look at the sizes of different body parts in *C. maculatus*, to see whether they too are trading off some against others. Like *P. interpunctella*, *C. maculatus* acquire all their resources during larval growth (Fox 1993), and live for relatively short times as adults (10 to 15 days in the moth (Gage 1995) and around seven to 20 days in the beetle (personal observation)), and mate multiple times. It is surprising that, given these similarities, larval density has the opposite effect on ejaculate allocation in the two insects. However, while *C. maculatus* larvae remain static within their bean hosts, *P. interpunctella* larvae are mobile and have physical contact with one another (Gage 1995); this might explain why male moths increase ejaculate allocation in response to high larval density, as they might be able to more effectively gauge population density and even population sex ratio, due to physical interactions with conspecifics. There is some evidence that *C. maculatus* larvae can detect the presence of other larvae sharing the same bean, by vibrations caused by larval chewing (Thanthianga and Mitchell 1987); if larvae were aware they were sharing beans, and consequently had an indication of increased population density or post-copulatory sexual selection level, they might be expected to increase investment in reproductive resources in attempt to more effectively engage in sperm competition. That my results find they do not, suggests either that bean-sharing does not indicate sperm competition risk, or that resource limitation is so severe that larvae are unable to effectively increase investment in reproduction, despite being able to detect conspecifics

developing in the same bean. When *P. interpunctella* larvae were deprived of sufficient nutrition (given only bran and glycerol), they produced fewer sperm than those also given yeast (Gage and Cook 1994). This result is comparable with mine, as my high density larvae likely suffered reduced nutritional resource availability as a result of having to share their bean substrate. In Gage's 1995 study, all larvae were given equal food irrespective of their density treatment; this might explain why in this case high density larvae produced more sperm – they were not nutritionally constrained, so were able to invest in sperm competition. In this case, larval density might therefore represent competition for mates rather than competition for resources. It would be interesting to carry out a study limiting food provisions to *P. interpunctella* larvae reared at different densities, to see at what point resource acquisition limits the increase in sperm numbers produced by males reared at high density.

Larval and early life conditions have been found to affect ejaculate allocation in a number of other insects, including the armyworm, *Pseudaletia separata* (He and Miyata 1997), the false garden mantid, *Pseudomantis albofimbriata* (Allen *et al* 2011) and the cockroach, *Nauphoeta cinerea* (Harris and Moore 2005). In the armyworm, larvae reared in groups produce more apyrene sperm than larvae reared solitarily (He and Miyata 1997), although there is no difference in eupyrene sperm numbers. Because apyrene sperm have been posited to assist mobility of eupyrene sperm within the female reproductive tract (He and Miyata 1997), it is thought this increase in their numbers is a reaction to the increased risk of sperm competition represented by group rearing. Although this contrasts with my result, armyworm larvae were provided with nutrition during both larval and adult life (He and Miyata 1997), which might explain why they were able to increase investment in sperm, while my *C. maculatus* males were not. Males of the false garden mantid produce more sperm when reared in a male-biased sex ratio as larvae than when reared in a female-biased sex ratio (Allen *et al* 2011). Males reared in male-biased conditions take longer to emerge as adults, but emerge at the same body size as males reared in female-biased conditions (Allen *et al* 2011), suggesting

body development might be delayed in order to invest more heavily in reproductive traits. I did not measure larval development time in my study; it would be interesting to investigate whether a longer development time could account for high larval density males achieving the same body size as low larval density males in *C. maculatus*. Male cockroaches housed in the presence of other individuals during sexual maturation produce larger spermatophores than those housed alone (Harris and Moore 2005). Because larger spermatophores delay female re-mating in this species (Harris and Moore 2005), this appears to be an adaptation to avoid sperm competition.

These studies demonstrate reproductive plasticity arising as a response to larval conditions, but few measured whether different male tactics resulted in the expected differences in male reproductive success; my findings in Chapter 3 suggest this is a necessary step before the behaviour can be considered adaptive. In my current study, male responses to larval conditions were proven to affect reproductive success; however, males in my experiment appear to be constrained by dense larval rearing conditions, rather than being able to react to sperm competition by increasing investment in reproductive traits. My results are therefore not consistent with sperm competition theory, which predicts males exposed to high levels of sperm competition should increase ejaculate allocation. My high larval density treatment should represent a high level of sperm competition because it predicts adult population density, and a more dense population should involve more competition for mates. In Chapter 3, I demonstrated that males did have the ability to plastically alter their ejaculate allocations in response to sperm competition levels, but the current findings suggest this ability might be constrained by harsh larval rearing conditions. It is possible that males that are not constrained as larvae retain behavioural plasticity as adults, but males limited by larval resource acquisition cannot – it would be interesting to subject males from both larval density treatments to different adult mating scenarios, to see whether they retain the ability to react plastically as adults to different sperm competition levels at different matings.

In summary, my findings suggest that male *C. maculatus* are constrained by conditions during larval growth, and high larval density leads to decreased ejaculate size and reproductive success for males. Males do not conform to sperm competition risk theory, which predicts those reared at high larval density should increase ejaculate allocation; either the harsh environment experienced by high larval density males renders them unable to invest in reproductive resources, or a high larval density is not in fact indicative of high levels of sperm competition in this species.

Chapter 5. Does larval density affect sperm numbers in *Callosobruchus maculatus*?

5.1. Introduction

In insects, ejaculate characteristics can affect male reproductive success in different ways. Larger ejaculates might contain more sperm, and so lead to greater success in sperm competition (Tomkins and Simmons 2000; Schaus and Sakaluk 2001; Engqvist *et al* 2007) or, if females delay subsequent copulations until their sperm stores are depleted, they might delay females re-mating with rivals, to limit the occurrence of sperm competition (Svärd and Wiklund 1989). Larger ejaculates might also contain a greater volume of other ejaculatory products, which could influence female behaviour (Rice 1996; Simmons and Siva-Jothy 1998; Moreau *et al* 2002; Fricke *et al* 2009). In *Callosobruchus maculatus*, sperm numbers have been shown to be important in determining success in sperm competition; when numbers of sperm inseminated by second-mating males were experimentally reduced, these males achieved lower reproductive success (Eady 1995). Larger numbers of sperm have also been shown to be associated with delayed female re-mating in *C. maculatus* (Eady 1995). Because sperm of previous males are physically displaced by second ejaculates in *C. maculatus*, leading to strong last-male fertilisation precedence (Eady 1994), a larger ejaculate inseminated by a second male should also increase his reproductive success, relative to that of his competitor. In addition, evidence that females might re-mate to obtain water (Edvardsson 2007) suggests males inseminating ejaculates bulked out with extra water could gain reproductive success by delaying female re-mating, thus avoiding sperm competition. Female longevity has been shown to be improved by mating multiply when nutrient-stressed (Fox 1993). Female re-mating is also delayed, and fecundity increased, by nutrient provision (Fox and Moya-Larańo 2009), suggesting investing in ejaculatory nutrients might increase male reproductive success.

These benefits of increased ejaculate allocation, together with the fact that a male's sperm reserves are finite (Wedell *et al* 2002), leads sperm competition theory to suggest that males should allocated sperm economically between different matings over their lifetime (Parker 1970), depending on sperm competition levels. In many insects, conditions experienced in early life can provide information about likely future conditions, including population density and sperm competition level. Therefore, males should be selected to adjust their sperm allocation depending on larval conditions. Previous work with various insects has been consistent with this theory (Gage 1995; He and Miyata 1997; Harris and Moore 2005; Yamane and Miyatake 2005; 2008; Allen *et al* 2011). In Chapter 4, I showed that *C. maculatus* males reared at high larval density produced smaller ejaculates and achieved lower reproductive success than males reared at low larval density; the opposite result to that predicted by sperm competition theory. However, I did not test which components of the ejaculate differed between males from different larval density treatments. In this chapter, I examine directly the effect of larval density on the number of sperm that males inseminate during their first mating, with the aim of understanding the causes of the changes in reproductive success found in Chapter 4.

Numerous studies have demonstrated that larval conditions can affect the numbers of sperm males allocate to matings (Gage 1995; He and Miyata 1997; Allen *et al* 2011; Harris and Moore 2005; Yamane and Miyatake 2005; 2008). Some have shown that males react to greater levels of sperm competition, represented by high larval densities, by increasing sperm numbers. Male *Plodia interpunctella* reared at high density inseminate more sperm during mating than males reared at low density (Gage 1995), and male *Pseudaletia separata* armyworm produce more apyrene sperm when reared at high larval density than when reared at low larval density (He and Miyata 1997). In these instances, males are reacting to the higher levels of sperm competition represented by more dense larval conditions, and are investing more in reproductive resources accordingly. Similarly, male *Nauphoeta cinerea* cockroaches housed with conspecifics during sexual development produce larger

spermatophores and more sperm than males housed alone (Harris and Moore 2005). Male *Pseudomantis albofimbriata* false garden mantids produce more sperm when reared in a male-biased sex ratio than when reared in female-biased conditions (Allen *et al* 2011); again males react to the higher risk of sperm competition, as indicated by the presence of male conspecifics, by increasing reproductive investment in order to engage in the competition. Despite these clear results, many of these studies controlled for larval food provisioning (Gage 1995) so, although they were subject to denser conditions, larvae did not face resource limitations. There is evidence in insects that limiting larval resource availability decreases survival (Giga and Smith 1981; 1991), body condition (Colegrave 1995) and reproductive success (Gage and Cook 1994); in the wild, it is likely high larval density goes hand in hand with resource limitation, certainly when larvae grow in closed systems.

Male *Callosobruchus chinensis* beetles reared at high larval density, within the closed system of a dried seed host, produce greater numbers of sperm than those reared at low larval density, when they belong to strains with high levels of polyandry (Yamane and Miyatake 2005; 2008). High larval density males react to the presence of other larvae by increasing the number of sperm they inseminate on mating, while males reared at low density perceive a low risk of sperm competition, so invest less in sperm. In contrast, males of monandrous strains, or strains with low levels of polyandry, produce fewer sperm when reared at high larval density than when reared at low larval density (Yamane and Miyatake 2008). In this case, populations have not been subjected to generations of polyandry, so males are not selected to react to sperm competition. High density males instead face resource limitation and consequently produce fewer sperm (Yamane and Miyatake 2008). In *C. chinensis*, therefore, it seems that larval density affects both perceived sperm competition level and resource availability, but that which force affects males more depends on the historic levels of polyandry in that population.

There is also evidence in some insects that male ejaculate allocation varies over different copulations (Svärd and Wiklund 1986; Cook and Wedell 1996). Male *Pieris rapae* butterflies inseminate greater numbers of sperm when mating for a second time (with a second female) than during their first mating (Cook and Wedell 1996), as sperm competition is likely to be a greater risk later in the season, when a female is less likely to be virgin. Conversely, male *Papilio machaon* butterflies inseminate most sperm during their first mating, and sperm number decreases with subsequent matings thereafter (Svärd and Wiklund 1986); in this instance, males decrease the number of sperm allocated to matings with non-virgins, possibly due to the diminishing returns of investing reproductive resources when sperm competition is intense (Parker 1970). Because larval conditions can indicate future conditions, it is possible adaptations to larval density go beyond a male's first mating and potentially last their lifetime, also influencing ejaculate allocations at subsequent matings, due to either perception of sperm competition level, or effects of resource limitation. In Chapter 4, I examined the effect of larval density on the ejaculate size and reproductive ability of a male's first ejaculate. In this chapter, I seek to investigate whether larval density in *Callosobruchus maculatus* can influence the sperm allocation of males, and their ability to fertilise a clutch of eggs after they have copulated a number of times.

In *C. maculatus*, it is known that larval density affects survival to adulthood (Mitchell 1975), and fitness (Giga and Smith 1991; Colegrave 1995); early conditions clearly influence adult life history. Chapter 4 investigated how larval density affected male body size, ejaculate size and male reproductive success, and found that when reared at high density, males produced smaller ejaculates and achieved lower reproductive success, but that body size was not affected. Here, I investigate how larval density affects the number of sperm males inseminate, to test whether larval density constrains sperm numbers or whether it is other components of the ejaculate that are altered in ejaculates of different size. This will help shed light on the relative importance of sperm and other ejaculatory components in determining male reproductive success – if I find larger ejaculates contain more sperm, it is likely these

are responsible for the elevated reproductive success of males from low larval density that I found in Chapter 4. To manipulate larval density, I rear larvae either solitarily or in groups of five, and measure weights of ejaculates produced by males from the different larval density treatments. I then count the number of sperm in samples of ejaculate from each male. I also subject a subset of these focal males to a number of sequential matings and, at their last mating, measure ejaculate weight, and count the number of fertilised eggs their female mates lay, to investigate whether the effects of larval density on ejaculate size are consistent across subsequent copulations, and whether male fertility after several matings is influenced by larval density.

5.2. Methods

To investigate the effect of larval density on sperm number and ejaculate size, males were reared at either high or low larval density in black-eyed beans; as adults they were mated, their ejaculate sizes were measured, and their sperm numbers counted. In addition, a subset of males was mated five times sequentially, and their ability to fertilise a clutch of eggs was measured in order to investigate whether males reared at different larval densities differ in the rate at which their ejaculatory resources deplete.

5.2.1. Manipulating larval density

Larval density was manipulated in the same way as in Chapter 4; either one larva per bean or five larvae per bean. Only black-eyed beans were used as hosts this time as they are easier to work with, and, because I saw an effect of these manipulations of larval density on ejaculate size and reproductive success in Chapter 4, I concluded these were effective manipulations and were likely to lead to measurable differences in ejaculate size again. In Chapter 4, there were no effects of bean type on ejaculate size or male reproductive success, so this time only one bean type was used.

Approximately 100 non-virgin adult male and female *Callosobruchus maculatus* of the Niamey strain were added to around 400 black-eyed beans (*Vigna unguiculata*), and were given four hours to lay eggs, after which time adults were anaesthetised using carbon dioxide gas and were removed. Beans were examined under a dissecting microscope, and the number of eggs on each observed. Those beans bearing five or fewer eggs were discarded, in attempt to standardise bean quality; females might lay fewer eggs on poor quality beans (Mitchell 1975), and those bearing six or more eggs were randomly assigned to one of two treatments – low or high larval density. Using a scalpel, eggs were scraped off beans to leave the appropriate number; beans in the low larval density treatment were left with one egg per bean, and those in the high larval density treatment were left with five eggs per bean. All scraping was carried out within six hours of egg laying because within a few days, eggs hatch and larvae enter beans (Howe and Currie 1964); by manipulating egg number early on, the number of larvae entering the bean could therefore be reliably controlled. This process was repeated on six consecutive days (six experimental blocks), so that laying time, and hence offspring emergence time, was staggered over a number of days, to make the experiment more manageable.

Beans were kept at around 30° C for three weeks, to allow larval development, after which time they were individually isolated in 1.5 ml Eppendorf tubes with ventilation holes, in order to prevent adults mating when they emerged. Low larval density beans, although only carrying one individual each, were isolated in the same way, in order to standardise larval experience. When emergence was imminent, beans were checked daily and emerged adults were sexed; all males were given individual identification numbers and females were discarded. When multiple individuals emerged from the same high larval density bean on the same day, they were excluded from the study; if they were of opposing sexes they might have mated, and even if they were all male their ejaculate allocation tactics might have been influenced by the presence of other adult rival males.

Males were weighed on day of emergence to the nearest 0.001 mg and were isolated individually for 48 hours, to allow their sperm stores to fully mature before mating (Savalli and Fox 1999). Virgin females were collected from Niamey stock population, given individual identification numbers, and randomly allocated to mate with males from either the low or high larval density treatment. Females were mated within 24 hours of emergence; they were temporarily anaesthetised using carbon dioxide gas and were weighed to the nearest 0.001 mg both before and after mating. Ejaculate size was estimated as female weight gain, which was calculated by subtracting initial female weight from final female weight (Savalli and Fox 1999). Matings were carried out individually in 55 mm Petri dishes at room temperature (around 20 °C). Any females having gained no weight (or having lost weight) during mating were considered to have not mated, and so were excluded from the study.

5.2.2. Counting sperm

When females had been mated and weighed, they were immediately transferred to individual 1.5 ml Eppendorf tubes and put in the freezer (at around -20° C), in order to kill them and immobilise the ejaculates they had been inseminated with during mating, so that all sperm could reliably be dissected out at a later date. Females remained in the freezer for between 14 and 30 days to allow the mating section of the experiment to be completed before the sperm counting section began.

Sperm counts were carried out in accordance with instruction from P E Eady and R Vasudev. During tuition, sperm counts were found to be highly repeatable between two experimenters (see Chapter 2 for further detail). Each dead female beetle was placed in a small droplet of insect ringer (pH 7) for five minutes, in order to soften the carcass to make it easier to manipulate. Thereafter, the female was moved to a clean Petri dish and observed under a dissecting microscope. Two pairs of watchmakers' forceps were used to position the female

on her back, and the front section of the body (head, thorax and legs) was removed and discarded, leaving only the pygidium. The pygidium was then squeezed with one pair of forceps, which opened the genital tract. The end of the second pair of forceps was then inserted into the open genital tract, and the conjoined bursa and spermatheca were grabbed and gently pulled out, and were isolated from other tissue. The rest of the beetle was discarded, and the bursa and spermatheca moved onto a clean area of the Petri dish. Using the forceps, the spermatheca end and any other waste tissue were pulled off and discarded, leaving only the intact bursa.

100 µl of 1 % biological detergent was pipetted onto a watch glass. The bursa was then added and the forceps used to macerate the bursa for three minutes, to release the sperm into the detergent. This was left for five minutes, to allow time for the detergent to break down bursa tissue, while leaving the sperm tails intact (sperm heads were also degraded by the detergent).

20 µl of this mixture was then drawn up and loaded onto a 1 mm^3 volume haemocytometer, with 25 squares on each side (10 µl was loaded into each side). This was observed under a phase contrast microscope at x40 magnification. Sperm have the appearance of short, dark hairs. The numbers of sperm in five of 25 squares on each side of the haemocytometer were counted (ten squares counted for each female in total); the four corner squares and the central square. When observing a square, the fine focus control was adjusted to view the full depth of field of the haemocytometer, so that no sperm were missed (not all sperm lay flat). When counting sperm per square, all sperm contained completely within the square were counted; sperm that lay over the boundary of a square were counted if they lay over the top or right-hand side boundary of the square, but not if they lay over the bottom or left-hand side boundary, in order that these sperm were not effectively counted twice.

A calculation was then carried out for each female, to estimate the number of sperm in a 1 mm^3 sample. The total numbers of sperm in the ten squares counted were summed, and then an average per square calculated. This number was then multiplied by 50, to give an estimate of the average number of sperm in the haemocytometer (25 squares on each side), and that number was multiplied by 1000, to estimate the number of sperm in the 1 mm^3 sample. The estimated sperm count for males from high and low larval density treatments were compared, in order to investigate whether larval density affects sperm number.

5.2.3. Measuring sperm depletion

A subset of males was mated sequentially several times following their initial matings - males were given 24 hours after their first mating, after which time they were mated to four females from the stock population, one at a time, at 30 minute intervals. All copulations were observed. Matings were carried out individually in 55 mm Petri dishes at room temperature (around 20 °C). The final female mates were virgins, and were weighed to the nearest 0.001 mg before and after mating. Ejaculate size was estimated by subtracting female weight before mating from female weight after mating (Savalli and Fox 1999). Males that did not mate during any one of the opportunities were removed from the study.

After being mated and weighed, females were transferred to individual 90 mm Petri dishes, containing approximately 100 black-eyed beans each, and were kept at around 30 °C. Females were allowed to oviposit until death. After the last female had died, beans were left for five days before eggs were counted, in order to give time for all fertile eggs to hatch; hatching can take up to a few days following egg-laying (Howe and Currie 1964). Beans were then observed under a dissecting microscope, and the eggs on them were assigned as either fertilised or unfertilised by their appearance, in accordance with methods described in Chapters 2 and 3. Eggs that had turned white had hatched, so were fertile, whereas those that remained pale and translucent had not hatched, so were not fertile. The number of fertile eggs

laid by each female was taken as an estimate of the fertilising ability of the male with which she mated. Fertility of males from both the high and the low larval density were compared, in order to investigate whether larval density affects the rate at which ejaculatory resources deplete with sequential copulations.

5.2.4. Statistical analyses

All analyses were carried out using General Linear Models in Minitab 15. Initially, all explanatory factors, covariates and interactions were included to produce a maximal model; non-significant interactions were then removed sequentially to leave the minimal model. Minimal models included all factors related to experimental design (e.g. larval density treatment and block), whether or not they significantly affected the response, and also any interactions that did significantly affect the response. All stated statistics are from minimal models. Sperm number and fertilised egg number were square-root transformed, in order that the data fitted with the assumption of a normal distribution.

Effects of all interactions were tested but, for simplicity, interaction results are only presented if they have significant effects or if they are relevant to questions being addressed.

5.3. Results

5.3.1. Larval density and male body size

Male body size at emergence was unaffected by larval density ($F_{1, 200} = 0.19$, $p = 0.662$); see Figure 5.1.

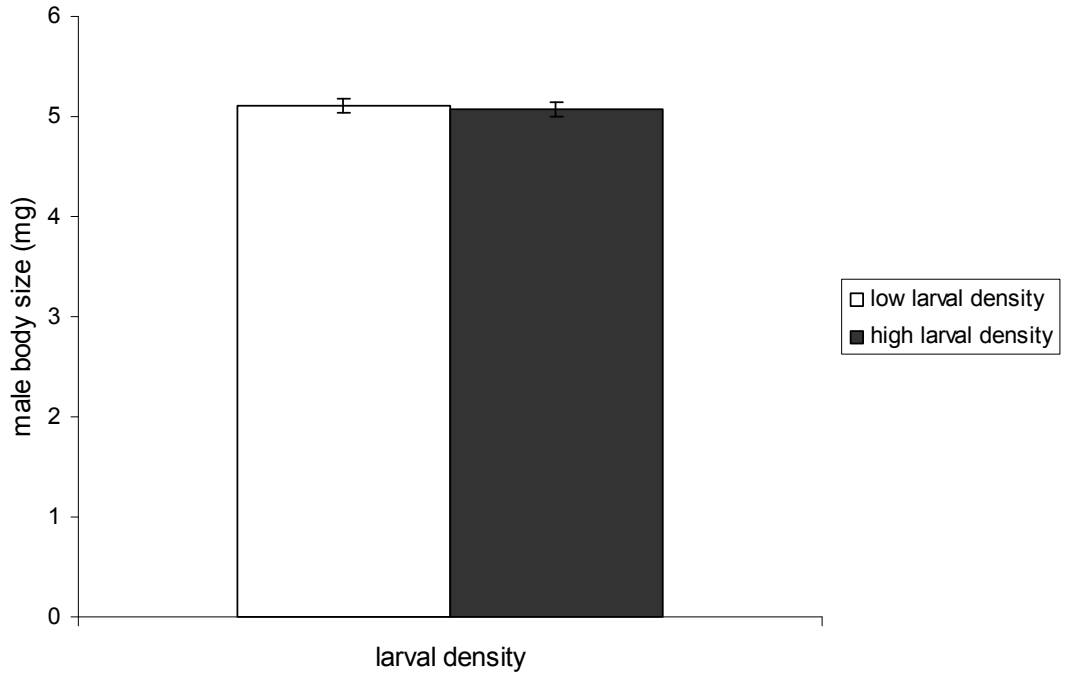

Figure 5.1: larval density and male body size. Male body size is given in milligrams, and is the mean value of males in each treatment group. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars show the standard error of the mean. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

Block did affect male body size ($F_{5, 200} = 7.79$, $p < 0.001$), and there was a borderline effect of the interaction between larval density and block ($F_{5, 195} = 2.15$, $p = 0.062$) - there was no consistent effect of larval density on male body size across blocks; see Figure 5.2.

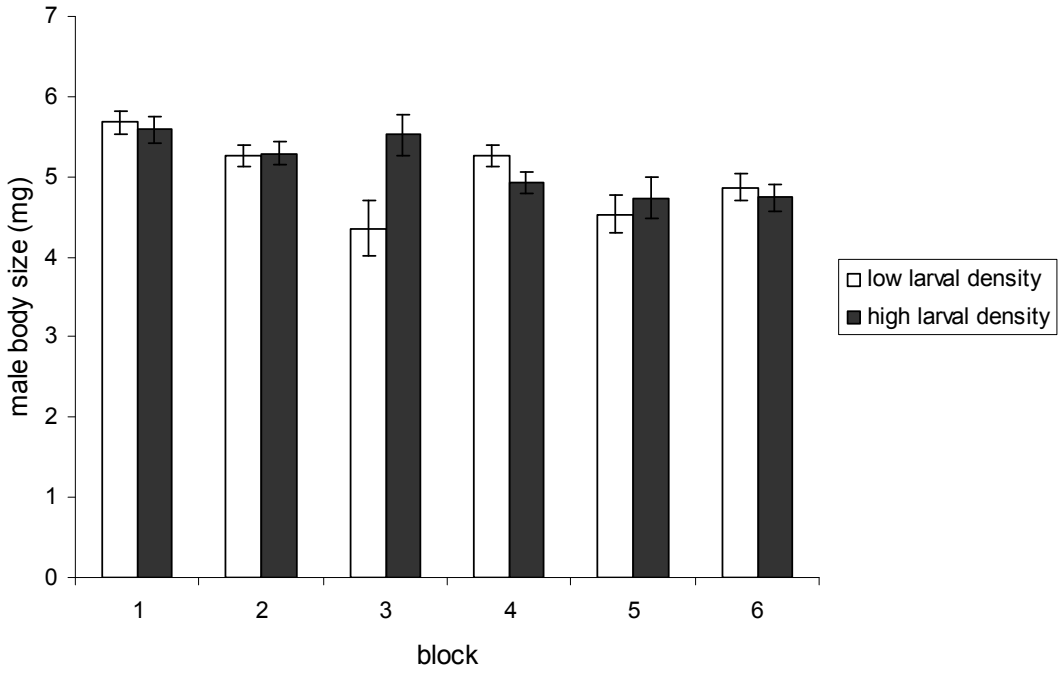

Figure 5.2: larval density, male body size and block. Male body size is given in milligrams and is the mean value of males in each treatment group, in each block. Empty bars represent males from low the larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. For block 1 low larval density males, n (sample size) = 23 and for high larval density males, n = 19; for block 2 low larval density males, n = 26 and for high density males, n = 25; for block 3 low larval density males, n = 4 and for high larval density males, n = 7; for block 4 low larval density males, n = 25 and for high larval density males, n = 28; for block 5 low larval density males, n = 9 and for high larval density males, n = 7; and for block 6 low larval density males, n = 16 and for high larval density males, n = 18.

5.3.2. Larval density and ejaculate size

Larval density affected ejaculate size ($F_{1, 200} = 24.02$, $p < 0.001$); see Figure 5.3. Males reared at low larval density produced ejaculates that were around 21.61 % larger than ejaculates produced by males reared at high larval density. There were no effects of block ($F_{5, 200} = 1.44$, $p = 0.210$).

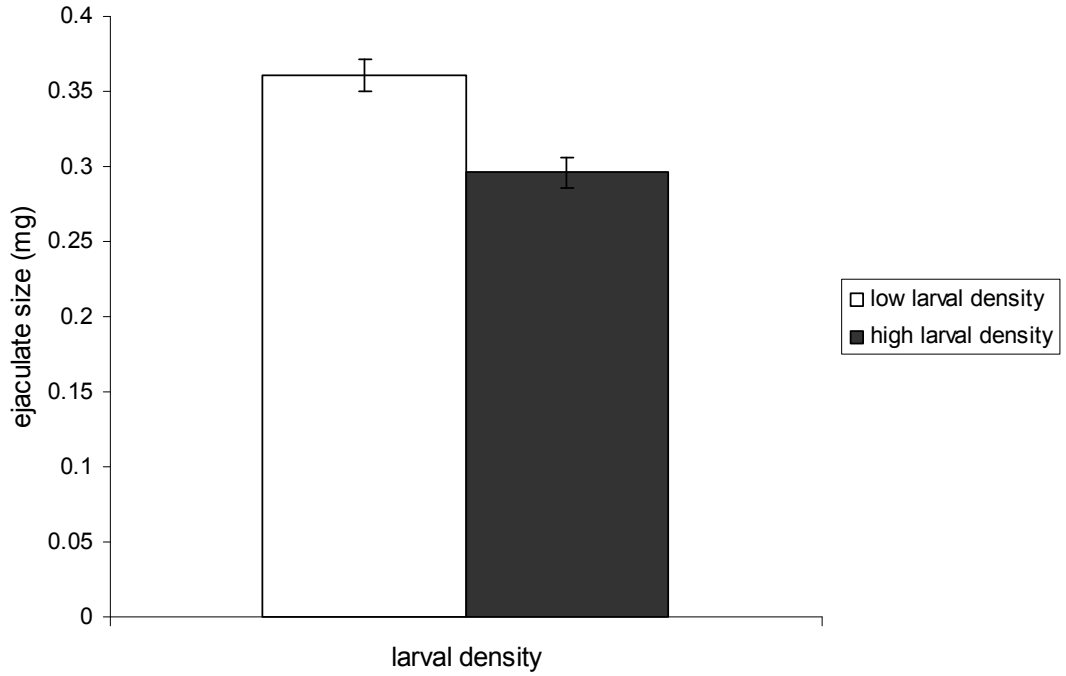

Figure 5.3: larval density and ejaculate size. Ejaculate size is given in milligrams, and is the mean value of males in each treatment group. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars show the standard error of the mean. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

Ejaculate size is known to be associated with body size in *C. maculatus* (Savalli and Fox 1999), so to control for this, the analysis was repeated with male body size added as a covariate. Indeed male body size did affect ejaculate size ($F_{1, 199} = 19.52$, $p < 0.001$); see Figure 5.4, but with male body size controlled for, the effect of larval density on ejaculate size remained significant ($F_{1, 199} = 24.83$, $p < 0.001$). Ejaculate size was also affected by block ($F_{5, 199} = 3.61$, $p = 0.004$), but there was no effect of the interaction between larval density and block ($F_{5, 194} = 0.69$, $p = 0.634$) - the size of the effect of larval density on ejaculate size differed between blocks, but the direction was always the same; see Figure 5.5.

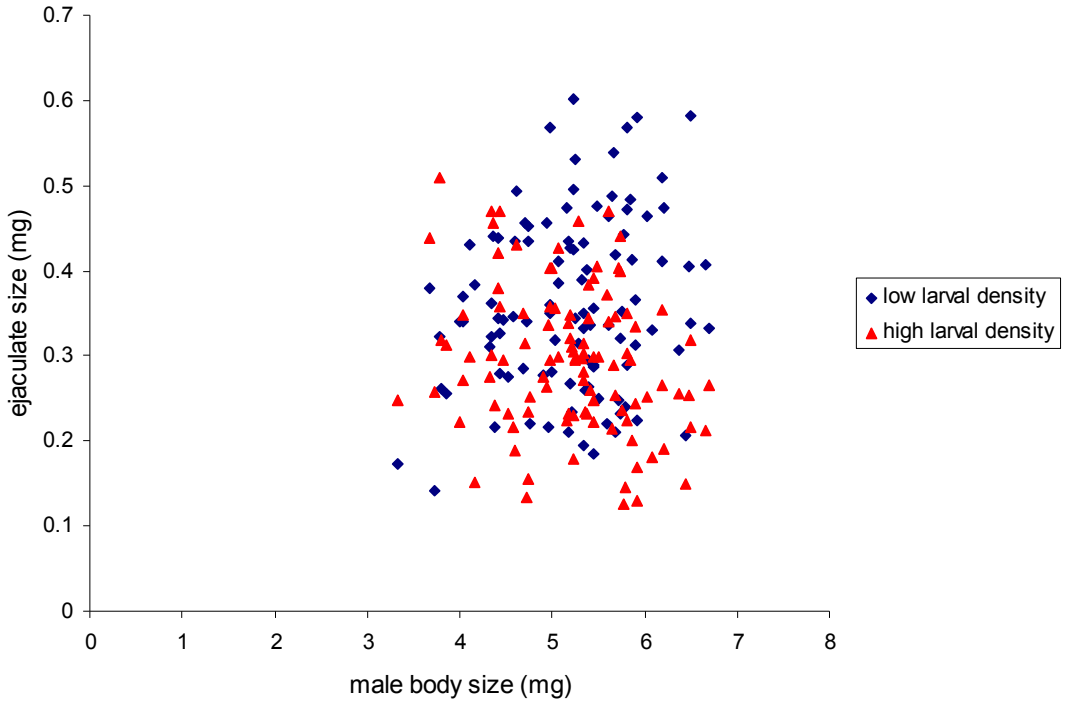

Figure 5.4: male body size and ejaculate size. Male body size and ejaculate size are given in milligrams. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

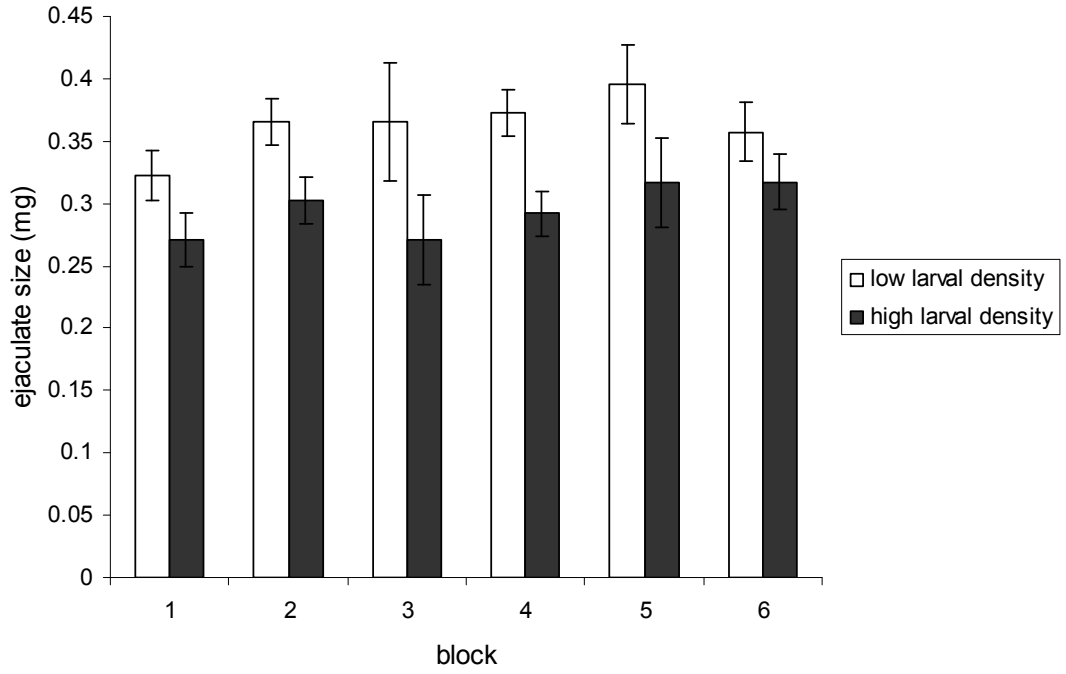

Figure 5.5: larval density, ejaculate size and block. Ejaculate size is given in milligrams and is the mean value of males in each treatment type, in each block. Empty bars represent males from the low larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. For block 1 low larval density males, n (sample size) = 23 and for high larval density males, n = 19; for block 2 low larval density males, n = 26 and for high density males, n = 25; for block 3 low larval density males, n = 4 and for high larval density males, n = 7; for block 4 low larval density males, n = 25 and for high larval density males, n = 28; for block 5 low larval density males, n = 9 and for high larval density males, n = 7; and for block 6 low larval density males, n = 16 and for high larval density males, n = 18.

5.3.3. Larval density and sperm number

To examine whether there was a general effect of larval density on sperm number, a general linear model was fitted with larval density and block as factors, and their interactions. Larval density affected the number of sperm inseminated by males ($F_{1, 200} = 9.77$, $p = 0.002$) – males reared at low larval density inseminated around 23.53 % more sperm than males reared at high larval density; see Figure 5.6. Sperm number was also affected by block ($F_{5, 200} = 2.64$, p

= 0.024), but there was no effect of the interaction between larval density and block ($F_{5, 195}$ = 0.47, p = 0.798) – the effect of larval density on sperm number did not differ significantly between blocks; see Figure 5.7.

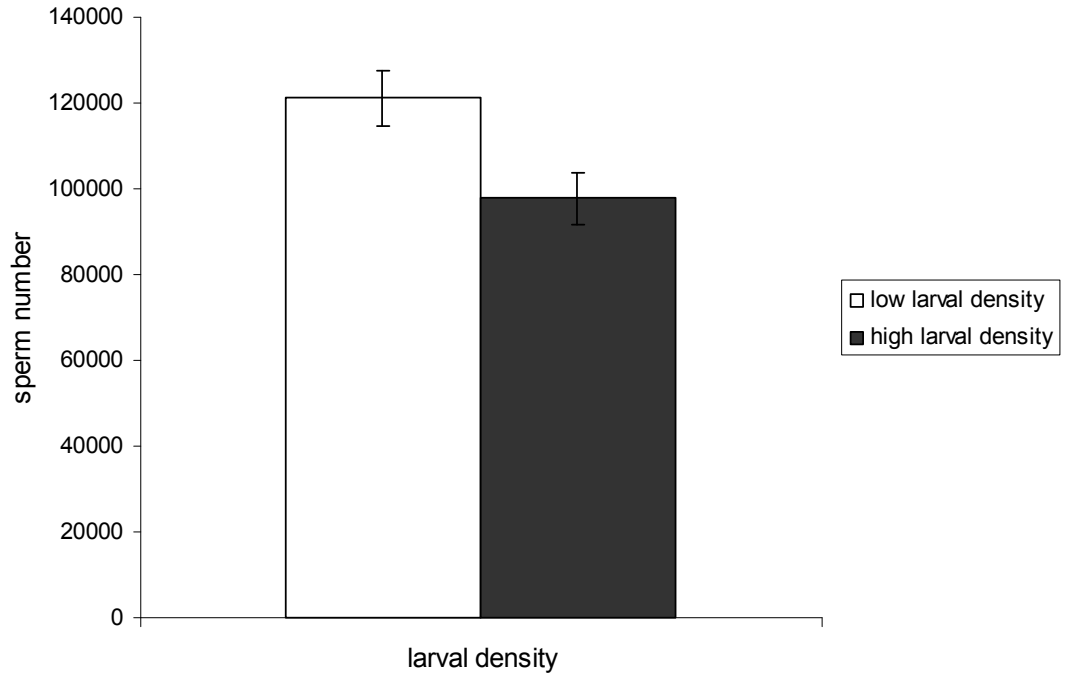

Figure 5.6: larval density and sperm number. Sperm numbers given are the mean values of males in each treatment group. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars show the standard error of the mean. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

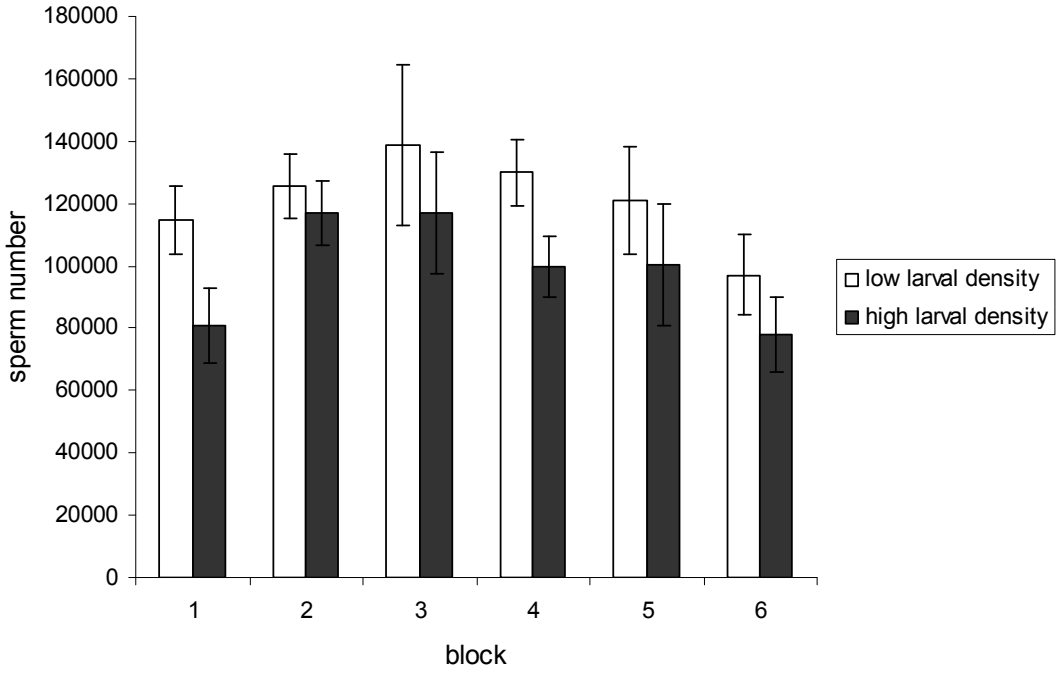

Figure 5.7: larval density, sperm number and block. Sperm numbers are given as the mean values of males in each treatment group, in each block. Empty bars represent males from the low larval density treatment and filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. For block 1 low larval density males, n (sample size) = 23 and for high larval density males, n = 19; for block 2 low larval density males, n = 26 and for high density males, n = 25; for block 3 low larval density males, n = 4 and for high larval density males, n = 7; for block 4 low larval density males, n = 25 and for high larval density males, n = 28; for block 5 low larval density males, n = 9 and for high larval density males, n = 7; and for block 6 low larval density males, n = 16 and for high larval density males, n = 18.

To control for potential effects of male body size on sperm number, the analysis was repeated and male body size was added as a covariate. Male size itself did not affect sperm number ($F_{1, 199} = 1.54$, $p = 0.216$); see Figure 5.8, and larval density retained a significant effect on sperm number, with male size controlled for ($F_{1, 199} = 9.54$, $p = 0.002$); males reared at low density produced around 23.07 % more sperm for their body size than males reared at high density did. Sperm number was affected by block ($F_{5, 199} = 2.56$, $p = 0.029$), but there was no effect of the interaction between larval density and block ($F_{5, 194} = 0.47$, $p = 0.800$).

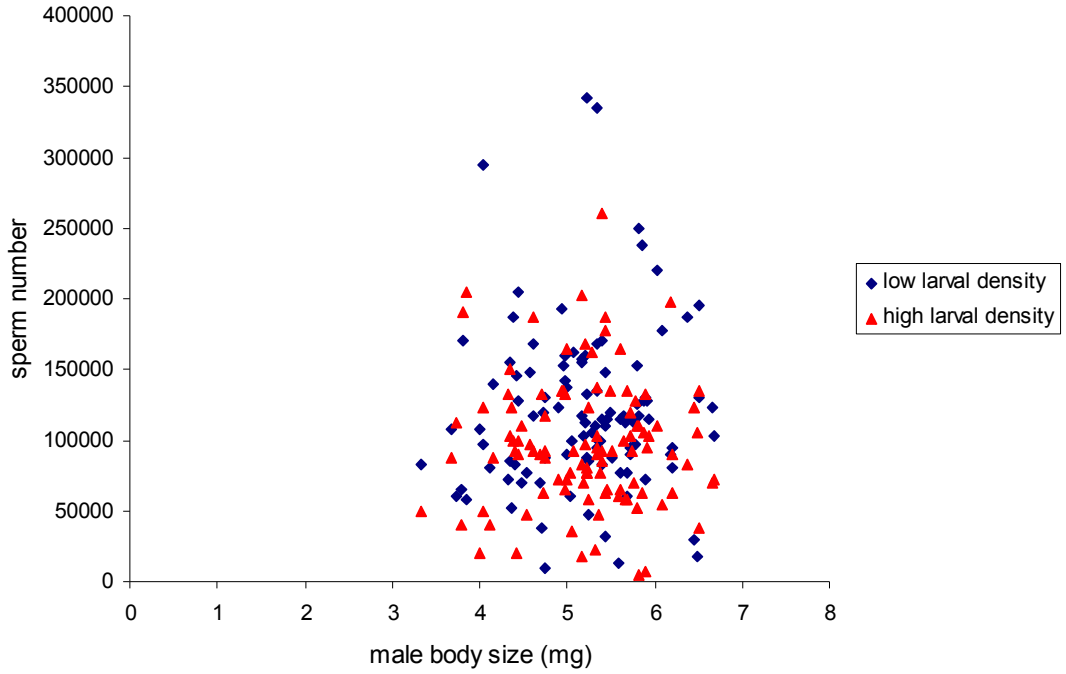

Figure 5.8: male body size and sperm number. Male body size is given in milligrams. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

One possible mechanism of the effect of larval density on sperm number is via its effect on ejaculate size (see Figure 5.3). To test this, the analysis was repeated but ejaculate size was added to the model as a second covariate. Larval density did affect sperm number ($F_{1, 198}$ = 4.21, p = 0.042), as did ejaculate size ($F_{1, 198}$ = 7.43, p = 0.007) - larger ejaculates contain more sperm; see Figure 5.9. For an ejaculate of a given size, males reared at low density inseminated around 15.29 % more sperm than males reared at high density. Sperm number was affected by block ($F_{5, 198}$ = 2.57, p = 0.028), but not by the interaction between larval density and block ($F_{5, 188}$ = 0.93, p = 0.465); the size of the effect of larval density on sperm number differed between blocks, but the direction was always the same. Sperm number was unaffected by male body size ($F_{1, 198}$ = 0.15, p = 0.698).

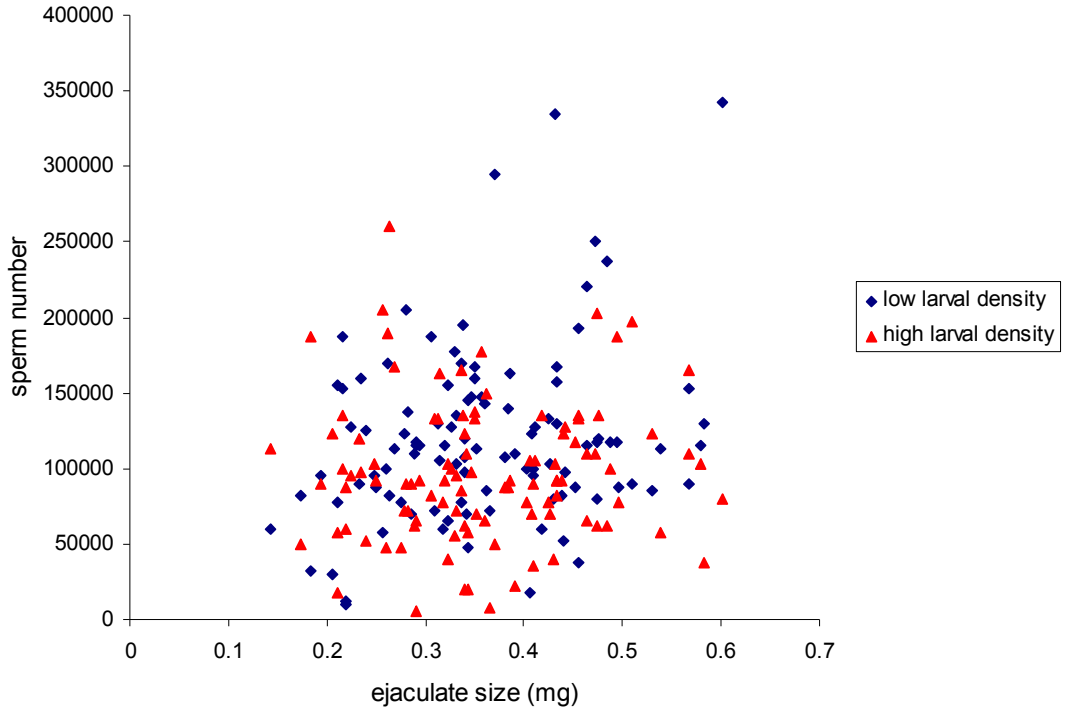

Figure 5.9: ejaculate size and sperm number. Ejaculate size is given in milligrams. Blue points represent males from the low larval density treatment and red points represent males from the high larval density treatment. Each data point represents one male. For low larval density males, n (sample size) = 103 and for high larval density males, n = 104.

The equation relating ejaculate size to sperm number in low larval density males is √sperm number = (207.74 x ejaculate size) + 261.54, and in high larval density males is √sperm number = (125.23 x ejaculate size) + 265.40. This suggests, in both cases, the relationship between ejaculate size and sperm number is non-linear; sperm number increases more rapidly as ejaculate size increases.

That larval density affects sperm number with ejaculate size controlled for in the model suggests larval density affects sperm number over and above its effect on ejaculate size, and affects sperm number corrected for male body size as well as affecting absolute sperm number.

5.3.4. Larval density and male fertility after sequential matings

To examine the effect of larval density on ejaculate size inseminated during the fifth mating, a simple general linear model was carried out with only larval density as a factor. Ejaculate size at fifth mating was not affected by larval density ($F_{1, 28} = 0.52$, $p = 0.477$); see Figure 5.10.

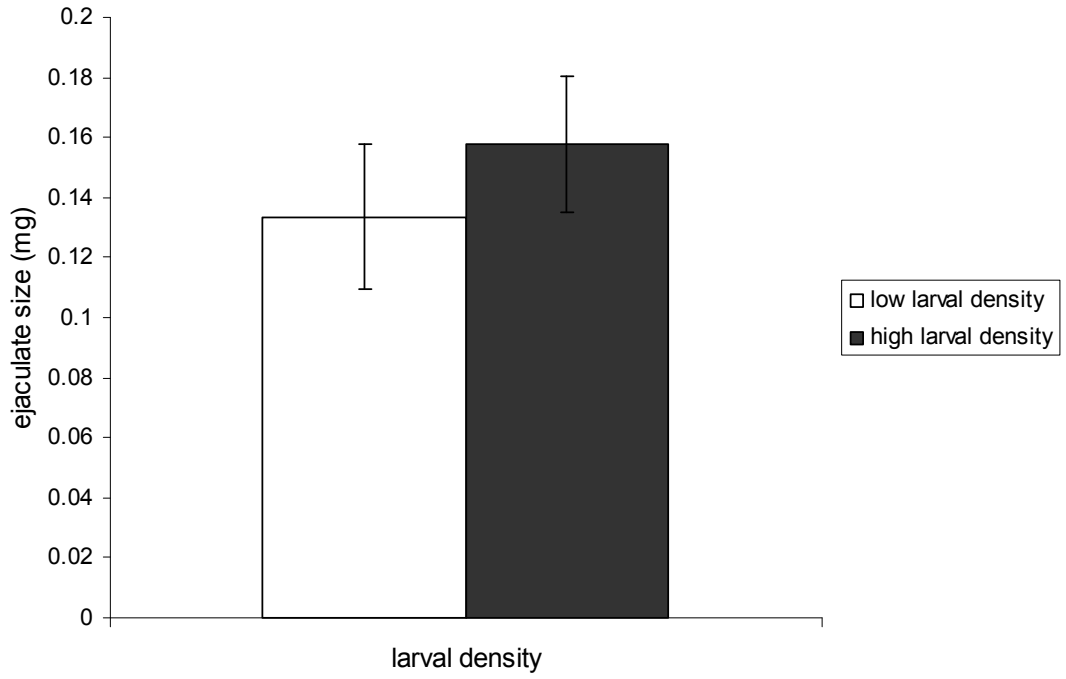

Figure 5.10: larval density and ejaculate size at fifth sequential mating. Ejaculate size is given in milligrams and is the mean value of males in each treatment group. The empty bar represents males from the low larval density treatment and the filled bars represent males from the high larval density treatment. Error bars show the standard error of the mean. For low larval density males, n (sample size) = 15 and for high larval density males, n = 15.

To investigate the effect of larval density on male fertilising ability after five sequential matings, the same simple model with only larval density as a factor was used. The number of

eggs fertilised by males at their fifth mating was unaffected by their larval density ($F_{1, 28}$ = 2.56, p = 0.121).

Ejaculate size can affect fertilising ability in *C. maculatus* (Savalli and Fox 1999); to control for this, the analysis was repeated but ejaculate size was added to the model as a covariate. Ejaculate size did have a borderline effect ($F_{1, 26}$ = 3.30, p = 0.081), and with ejaculate size controlled for, larval density did affect the number of eggs fertilised by males ($F_{1, 26}$ = 10.10, p = 0.004) – males reared at low larval density fertilised around 25.91 % more eggs than males reared at high larval density; see Figure 5.11. There was also an effect of the interaction between larval density and ejaculate size ($F_{1, 26}$ = 6.17, p = 0.020), suggesting ejaculate size affected male fertilising ability differently in the two larval density treatments. However, there was no obvious relationship between ejaculate size and fertility in males from either larval density treatment.

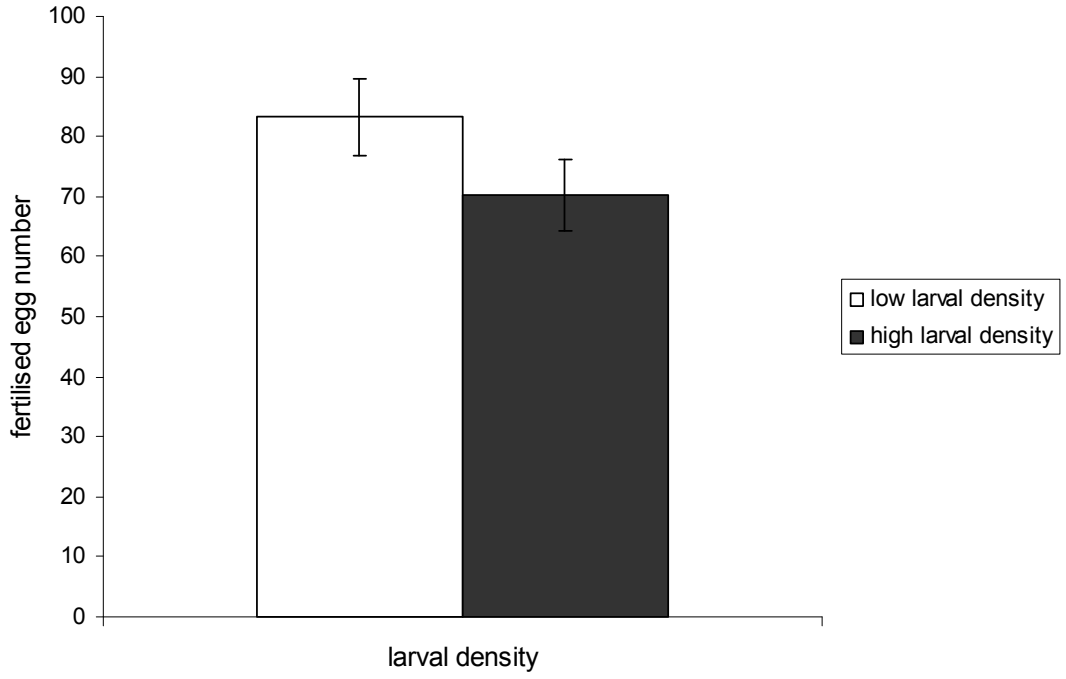

Figure 5.11: larval density and male fertility at fifth sequential mating. Fertilised egg numbers given are the mean value of males in each treatment group. The empty bar represents males from the low larval density treatment and the filled bar represents males from the high larval density treatment. Error bars give the standard error of the mean. For low larval density males, n (sample size) = 15 and for high larval density males, n = 15.

5.4. Discussion

Male *C. maculatus* reared at low larval density produced larger ejaculates and more sperm than those reared at high larval density. Male body size was unaffected by larval density, but controlling for it in analyses revealed males reared at low density produced larger ejaculates and more sperm for their body size than did males reared at high larval density. The definite effect of larval density on ejaculate size gives strength to the borderline effect found in Chapter 4; taken together, the chapter results suggest the ejaculate size effect is reliable. The larger ejaculates produced by males reared at low larval density also contained more sperm per volume of ejaculate than those produced by males reared at high larval density, as evidenced by the significant effect of larval density on sperm number when ejaculate size was

controlled for in analyses. This makes sense in light of the results from Chapter 4, in which larval density affected male reproductive success directly as well as its effect via ejaculate size, and suggests sperm numbers within ejaculates might be responsible for the direct effect of larval density on reproductive success. The size of the direct effect of larval density on sperm number (a difference of 15.29 %), compared to the size of its effect via ejaculate size (a difference of 23.53 %), suggests the majority of the overall effect of larval density on sperm number is due to direct effects, rather than effects via ejaculate size i.e. sperm numbers produced by low density males are greater than would be expected if the relationship between ejaculate size and sperm number was linear. There are reasons to expect high larval density to lead to lower sperm numbers in this species, because larval crowding has been previously shown to affect survival to adulthood (Mitchell 1975) and fitness (Giga and Smith 1991; Colegrave 1995). Similarly, resource limitation during larval growth has been shown to decrease sperm numbers in other species (Gage and Cook 1994).

However, theory suggests males exposed to high risks of sperm competition should increase the number of sperm allocated to matings, in attempt to more effectively engage in the competition (Parker 1970), and evidence from other species supports this theory (Gage 1995; He and Miyata 1997; Harris and Moore 2005; Yamane and Miyatake 2005; 2008; Allen *et al* 2011). My findings in Chapter 3 suggest my male *C. maculatus* do have the ability to react plastically to sperm competition by altering ejaculate allocation, but results in Chapter 4, corroborated by this chapter, suggest this ability is not being exhibited this time. It could be that high larval density constrains males, so they are physically or physiologically unable to react to sperm competition, or that in *C. maculatus* larval density is not a good indicator of sperm competition level. This is surprising, given the opposite results found in the closely related *C. chinensis*, in which males of polyandrous strains reared at high larval density produced more sperm than those reared at low density (Yamane and Miyatake 2008). In the same study, it was found *C. chinensis* males of monandrous strains behave differently – males reared at high density produce fewer sperm than those reared at low density (Yamane and

Miyatake 2008). My results are consistent with this, however my *C. maculatus* population is polyandrous so my males would be expected to be selected to react to sperm competition indicators in the same way that polyandrous *C. chinensis* males do. It seems that *C. chinensis* males perceive high larval density as indicative of a high risk of sperm competition, and increase sperm numbers accordingly, whereas *C. maculatus* males are constrained at high larval densities and consequently produce fewer sperm than those males reared free from competition for resources. Possible reasons for these contrasting findings are differences in experimental design – in my experiment, males were 48 hours old when they mated, whereas the *C. chinensis* males were under 24 hours old; males in my experiment were housed alone before they mated whereas some *C. chinensis* males were held in groups; and I used black-eyed beans (*Vigna unguiculata*) as larval hosts whereas *C. chinensis* males developed in adzuki beans (*V. angularis*) (Yamane and Miyatake 2008). Another inconsistency when comparing results is that, whereas in *C. chinensis* males did emerge smaller from high density treatments (Yamane and Miyatake 2008), I found no difference in body size between male *C. maculatus* reared at high or low larval density. This is surprising, given previous evidence in this species (Giga and Smith 1991; Colegrave 1995; Gay *et al* 2009), and because the smaller ejaculates and sperm numbers are suggestive of physical or morphological constraints due to resource limitation – I would also expect this limitation to constrain body size. However, in some other species, despite adopting different sperm allocation tactics, males reared at different larval densities emerged with equal body sizes (Gage 1995; Allen *et al* 2011). It might be that if a larva can detect the presence of a competitor within its bean host, it diverts resources into body growth and away from investment in reproduction; this would explain why males emerge at a size equal to that of those developing solitarily in beans, but their ejaculate sizes and sperm numbers are smaller. This is supported by the finding that when body size was controlled for, low larval density males still produced more sperm (and achieved greater paternity, in Chapter 4). If males do trade off investment in reproduction against body size, this suggests body size is more important in this species than investment in reproductive resources, possibly due to the difficulty of locating and chasing female mates

(personal observation), and of achieving copulations when being kicked by females (Crudgington and Siva-Jothy 2000). It would be interesting to measure sizes of different body parts (e.g. testes) in my *C. maculatus* population, to see whether growth of some parts are traded off against growth of others, depending on larval density. Testes size is affected by larval conditions in some other insects (Gage 1995), but there is evidence of no effect on testes size in *C. maculatus* (Gay *et al* 2009). It is interesting that, in my study, larval density affected male reproduction independent of its effect on body size, whereas in female *C. maculatus* the opposite has been found (Colegrave 1993); larval density affects female fecundity, but only via its effects on female body size.

Another possible explanation for the lack of effect of larval density on male body size in my study is that high larval density might have lead to the death of weaker males before they emerged as adults, so males that do emerge from high density beans are the successful competitors, which are likely to be larger. When compared with males developing alone in beans, these males have equal body sizes, but, weaker competitors, which perhaps did not survive to emergence, might have been smaller. This would be consistent with other studies that found that mortality increased with increased larval density in *C. maculatus* (Mitchell 1975; Giga and Smith 1981; 1991). That the males in my study that did emerge from high larval density beans still produced smaller ejaculates and fewer sperm than those from low density beans, despite achieving equal body size, suggests my sperm number result is conservative; less fit high density males, if they had survived to emergence, might have produced even fewer sperm.

I found a significant effect of ejaculate size on sperm number – larger ejaculates contained more sperm. Interestingly, results suggested a non-linear relationship between ejaculate size and sperm number, with more rapid increases in sperm numbers as ejaculates get larger. This is surprising, as it might be expected there would be a limited number of sperm that could be allocated to a single ejaculate, and that after a certain ejaculate size is achieved, sperm

numbers plateau. That this is not the case suggests small ejaculates might be very constrained in the number of sperm they contain and that, rather than maximising sperm number in a minimally-sized ejaculate, males facing resource limitation allocate low numbers of sperm and relatively more of other ejaculatory products, while males with unlimited resources allocate large numbers of sperm and relatively less of other substances. It would be interesting to investigate the identities of other components within the ejaculates of *C. maculatus*, to see what other substances are invested in ejaculates, and whether they might act to affect female behaviour, as is the case in other species (Rice 1996; Simmons and Siva-Jothy 1998; Moreau *et al* 2002; Fricke *et al* 2009). My findings, together with those from Chapter 4, are consistent with other studies in *C. maculatus*, that greater numbers of sperm are associated with greater male reproductive success (Eady 1994; 1995). In Chapter 4, I found low larval density males produced larger ejaculates and achieved greater reproductive successes; in this chapter, I find again that low larval density males produce larger ejaculates, and that they produce more sperm. I can therefore conclude that the larger ejaculates and greater sperm numbers produced by low larval density males probably lead to their greater reproductive success when mating under competitive circumstances. In both this chapter and Chapter 4, ejaculates were of comparable sizes (in Chapter 4, a mean of 0.280 mg from low density males and a mean of 0.252 mg from high density males, and in this chapter, a mean of 0.359 mg from low density males and 0.295 mg from high density males). Again, this gives strength to the conclusion that the greater reproductive success of males in Chapter 4 were likely due to the greater numbers of sperm they produced, as demonstrated in this chapter.

Sperm competition theory suggests that, in order to maximise sperm number, under high sperm competition risk conditions, sperm size should be reduced (Parker 1970). There is some suggestion that sperm size could influence male reproductive success in some insects (Parker 1993; Gage 1994; Rugman-Jones and Eady 2008). In *Plodia interpunctella* moths, sperm length is maintained at the expense of sperm number when larvae grown under nutritional stress (Gage and Cook 1994), suggesting in this instance that sperm length is

actually more critical to reproductive success than gamete number, contrasting with predictions of sperm competition theory. In *C. maculatus*, the effect of sperm length on male reproductive success has not been examined, although there is evidence that sperm length has evolved in response to the female reproductive environment (Rugman-Jones and Eady 2008). Larval density does not affect sperm length in *C. maculatus* (Gay *et al* 2009), although maternal age does – sons of older mothers produce longer sperm (Gay *et al* 2009). Since the relationship between ejaculate size and sperm number in my study is non-linear (and because I did see an effect of larval density on reproductive allocation, but the Gay *et al* study did not), it would be interesting to measure sperm length to see whether it differs between males from the two larval density treatments in my population.

Despite inseminating ejaculates of the same size as those produced by low larval density males when mating for the fifth sequential time, males reared at high larval density fertilised fewer eggs during their fifth copulation. This suggests larval density affects not only a male's first mating, but has lasting effects over subsequent matings. Male ejaculate allocation has been shown to change over different copulations throughout male lifetimes in other insects (Cook and Wedell 1996). In my study, although fifth ejaculate sizes were not measurably different, they likely contained different numbers of sperm, evidenced by the different fertilising abilities of the ejaculates from males from low and high larval densities. The difference in numbers of eggs fertilised is not surprising, given the finding that sperm numbers in their first ejaculates are lower in high larval density males than in low larval density males – the lower fertilising ability of these males after sequential matings suggests they have smaller sperm numbers for life, and they might become sperm limited sooner than males reared at low larval density. It is interesting that there seemed to be a difference in the effect of fifth ejaculate size on the number of eggs fertilised within larval density treatments – there was a significant effect of the interaction between larval density and ejaculate size on male fertilising ability. Although there was no obvious direction of the relationship between ejaculate size and fertilising ability in males from either treatment, this could be a fruitful

course for future studies. It might be that the way males utilise their sperm stores, and other ejaculatory resources, differs depending on their larval conditions. Perhaps larval conditions cause some males to up-regulate other non-sperm ejaculatory components when they are sperm limited, whereas others do not, so ejaculates might remain of equal size but have differing fertilising abilities. Ejaculates at fifth matings are much smaller than initial ejaculates (means of 0.146 mg and 0.327 mg, respectively), so it might be that there is a difference in fifth ejaculate size between males from low and high larval densities, but it is too small to detect. It would be interesting to investigate this further by repeating the larval density manipulation and measuring sperm number as well as ejaculate size in males after a number of sequential matings.

In summary, I found that male *C. maculatus* are constrained by high larval density during development, and consequently produce smaller ejaculates containing fewer sperm. Taken together with findings from Chapter 4, I can conclude that these differences in sperm numbers and ejaculate sizes are most likely responsible for corresponding differences in male reproductive success. This is not consistent with sperm competition theory; either high density males are so limited by resources that they are unable to react to sperm competition, or larval density is not a reliable indicator of sperm competition level in this species. Because in Chapter 3 I found ejaculate size differences (in response to adult social context) that did not lead to differences in male reproductive success, I cannot assume that ejaculate size always corresponds to sperm number in this species, but, when caused by differences in larval density, it seems ejaculate size is a good indicator of sperm number. It might be that different components of the ejaculate are altered when males experience sperm competition as adults and as larvae. Differences in sperm number due to larval density also affect males throughout their lifetimes, and not just at their first copulation; males reared at high density become sperm-limited sooner, and are less able than males reared at low density to fertilise a complete clutch after a number of sequential matings.

Chapter 6. The effects of water provision on ejaculate size in *Callosobruchus maculatus*

6.1. Introduction

Ejaculate size has been shown to influence male reproductive success in a variety of insects (Tomkins and Simmons 2000; Schaus and Sakaluk 2001; Engqvist *et al* 2007). In some cases, larger ejaculates affect female oviposition or re-mating behaviour (Svärd and Wiklund 1989; Rice 1996; Simmons and Siva-Jothy 1998; Savalli and Fox 1999; Prout and Clark 2000), and in others, large ejaculates can displace or eject inseminations from other rival males (Danielsson 1998). This allows males to avoid, or more effectively engage in, sperm competition. In some insects, female re-mating is delayed by a large volume of ejaculate activating stretch receptors in female sperm-storage organs (Svärd and Wiklund 1989). In Chapter 4, I showed male *Callosobruchus maculatus* producing larger ejaculates as a result of favourable larval conditions achieved greater reproductive successes than other males that produced smaller ejaculates. In Chapter 5, I found that these larger ejaculates contained greater numbers of sperm, therefore suggesting more sperm might be at least partially responsible for larger ejaculates and greater reproductive success in *C. maculatus*. Because larval rearing conditions influence the quantity of resources individuals can acquire during growth and development, it is possible that sperm are not the only constituents that differ in quantity in ejaculates of different size. Normally, *C. maculatus* individuals do not eat or drink as adults, so the resources they acquire within their bean hosts as larvae are critical in determining their reproductive investment for life. Larvae having to share hosts with conspecifics might also be constrained in the quantity of water and other nutrients they can acquire.

The provisioning by males of nuptial gifts (nutrients or water, along with the sperm-containing ejaculate) to females during mating has been demonstrated in a number of insects (Vahed 1998; Gwynne 2008), and can function as either mating effort (in attempt to increase

the number of eggs fertilised by that male), or as paternal investment (in attempt to increase the number of offspring a female produces), or both. The value to females of these nuptial gifts can depend on their own body condition (Fox 1993), particularly when mating also confers costs, as is the case in *C. maculatus* (Edvardsson 2007), in which harmful spines on male intromittent organs puncture females internally during copulation (Crudgington and Siva-Jothy 2000; Edvardsson and Tregenza 2005). Female fecundity in *C. maculatus* has been shown to be lower in females that are prevented from kicking males off during copulation and therefore copulate for longer (Edvardsson and Tregenza 2005), highlighting the cost of mating. If females are in poor condition and lacking in nutrition, the benefit of receiving a nuptial gift might outweigh the cost of mating. Consequently, a male providing a large nuptial gift might benefit if his female mate has no need to mate again to gain resources, as he will avoid sperm competition, and because most of the offspring the female produces will be fathered by him. Furthermore, he will only be investing nutritionally in his own offspring and not those fathered by rival males. Indeed, in *C. maculatus*, females do take longer to re-mate when inseminated with larger ejaculates (Eady 1994; 1995) - as well as increased sperm numbers, this could be due to the provision of water or other nutrients in ejaculates; females receiving these in large quantities might have little need to re-mate, especially since in this species females generally acquire all the sperm they need to fertilise their lifetime supply of eggs during a single mating (Eady 1995). Because in the wild *C. maculatus* live in hot, dry areas, gaining water through ejaculate inseminations might increase female fitness, and because males do inseminate large ejaculates (up to 10 % of their body weight (Savalli and Fox 1998)), this suggests they have been selected to do so due to fitness benefits relating to ejaculate volume.

There is evidence in *C. maculatus* that females re-mate to obtain water (Edvardsson 2007; Fox and Moya-Larano 2009); therefore males inseminating more water with their ejaculates might delay female re-mating, and consequently achieve greater reproductive success by avoiding sperm competition. Because it is not possible to determine whether individuals

acquire different quantities of water from different larval condition treatments, in this chapter I subject adult males and females (having experienced the same larval conditions) to different water provision treatments, and measure the sizes of the ejaculates inseminated during matings. This could help determine whether water content can be responsible for size differences in ejaculates, and whether males differentially allocate water to copulations depending on the hydration level of their female mates. Water provision has been shown to affect ejaculate size and mating effort in some other insects (Ivy *et al* 1999).

Female *C. maculatus* benefit from being provided with water to drink as adults (Edvardsson 2007; Fox and Moya-Larano 2009); females given water live longer and produce more offspring than females denied water (Edvardsson 2007; but see Fox and Moya-Larano 2009). In addition, females given water are less likely to re-mate 24 hours after an initial mating (Edvardsson 2007; Fox and Moya-Larano 2009), and mate fewer times in total over their lifetimes, than females denied water (Edvardsson 2007). This suggests females gain material benefits from receiving water; if they cannot gain water through other means, acquiring it via ejaculates might confer the same benefits. Males could therefore benefit by increasing the water provision in their ejaculates so that females produce more of their offspring, and are less likely to re-mate with rivals (Edvardsson 2007; Fox and Moya-Larano 2009). Sugar has also been shown to affect female fecundity and behaviour (Fox 1993; Fox and Moya-Larano 2009) - females provided with sugar live longer, produce more offspring and re-mate less quickly than females provided with only water, or denied both water and sugar (Fox and Moya-Larano 2009). Also, females denied nutrition as adults live longer if they mate multiply, whereas females provided with sugar-water and yeast do not benefit from multiple matings in this way (Fox 1993). This suggests nutrition gained via ejaculates leads to increased longevity in females if they are nutrient-stressed (Fox 1993), as is likely in natural wild (and laboratory) populations, where food and water resources are generally lacking.

Chapters 4 and 5 investigated effects on ejaculate sizes and sperm numbers of different larval conditions experienced by males. Here, I subject adult male and female *C. maculatus* to different water provision treatments - either hydrated or non-hydrated, and measure ejaculate sizes, to investigate whether manipulating resources gained as adults affects ejaculate size. The experimental design is cross-factored; two male treatments and two female treatments, with matings in all combinations. It is therefore possible to investigate whether males produce ejaculates of different size depending on whether they acquire water as adults, and also to examine whether males allocate differently-sized ejaculates to females depending on female hydration.

6.2. Methods

To investigate the effect of giving water to both males and their female mates on ejaculate size in *Callosobruchus maculatus*, males and females were either provided with water (hydrated) or denied water (non-hydrated) as adults. Males of both treatments were mated to either hydrated or non-hydrated females, and their ejaculate sizes were measured.

6.2.1. Manipulating hydration

Virgin males and females of the Campinas strain of *C. maculatus* were isolated from stock population, and on emergence were given individual identification numbers. Beetles were randomly allocated to one of two treatments within their sexes – hydrated or non-hydrated, and were all housed in individual 55 mm Petri dishes. Those allocated to the hydrated treatment were each provided (in their Petri dishes) with an open 1.5 ml Eppendorf tube filled with water and plugged with a soaked ball of cotton wool. *C. maculatus* have been previously shown to drink water from this set-up (Edvardsson 2007). This volume of water was sufficient for individuals to be able to drink *ad libitum* for the duration of the treatment, without running out. Those individuals allocated to the non-hydrated treatment were not

provided with any water. All individuals remained in their treatments for 48 hours, at approximately 30 °C.

Males were then randomly allocated to females of either the hydrated or non-hydrated treatments, and were mated in individual 55 mm Petri dishes at room temperature (around 20 °C). Females were temporarily anaesthetised and weighed to the nearest 0.001 mg, both before and after mating, and ejaculate size was estimated as female weight gain (calculated by subtracting weight before from weight after mating). Females were then discarded, and males were returned to their labelled Petri dishes. Males were then once again left in their hydrated or non-hydrated treatments (males in the hydrated treatment had their water replenished) for 48 hours. During this 48 hours, a second round of newly-emerged virgin females were subjected to either the hydrated or non-hydrated treatment, as previously described. After 48 hours, males were offered their second mating with these new females (each male mated with females of the same hydration treatment for all three matings). Copulation was observed, and ejaculate size was measured. This process was repeated for a third round, so that in total each male mated three times with a virgin female, with 48 hours between each mating, during which the treatment (hydrated or non-hydrated) was maintained. Any males that failed to mate during any of the three mating opportunities were removed from the study.

Three measurements of ejaculate size were obtained for each male (one measurement at each mating); these were compared between males from the hydrated and the non-hydrated treatments, to investigate whether hydration level affects ejaculate size and the rate of decrease in size of ejaculates over sequential matings.

6.2.2. Statistical analyses

Statistical analyses were carried out using General Linear Models in Minitab 15. Initially, all explanatory factors, covariates and interactions were included to produce a maximal model;

non-significant terms were then removed sequentially to leave the minimal model. All stated statistics are from minimal models. Since the three measurements for each male are not independent, and each male is only in one combination of male and female hydration treatments, male ID was also included in statistical models as a random factor nested within male and female treatments.

Analyses revealed no interactions were significant; for simplicity, results of interactions are only presented if they are relevant to the questions being addressed.

6.3. Results

Male hydration affected ejaculate size ($F_{1, 72} = 17.11$, $p < 0.001$) – hydrated males produced ejaculates that were around 25.27 % larger than those produced by non-hydrated males. Ejaculate size was also affected by female hydration ($F_{1, 72} = 11.46$, $p = 0.002$) – males that mated with non-hydrated females inseminated ejaculates that were around 20.27 % larger than males that mated with hydrated females; see Figure 6.1. Largest ejaculates were given by hydrated males to non-hydrated females and smallest ejaculates were given by non-hydrated males to hydrated females. Ejaculates given by hydrated males to hydrated females, and by non-hydrated males to non-hydrated females, were intermediate in size (see Figure 6.1).

There was no effect on ejaculate size of the interaction between male hydration and female hydration ($F_{1, 72} = 1.25$, $p = 0.273$). Males inseminated larger ejaculates when mating with non-hydrated females, regardless of their own hydration treatment, and hydrated males produced larger ejaculates than non-hydrated males, regardless of whether they were mating with hydrated or non-hydrated females.

Ejaculate size was also affected by mating number ($F_{2, 72} = 30.25$, $p < 0.001$) – ejaculate size declined with each of three sequential matings; see Figure 6.1. Second ejaculates were around 30.21 % smaller than first ejaculates, and third ejaculates were around 8.38 % smaller than second ejaculates.

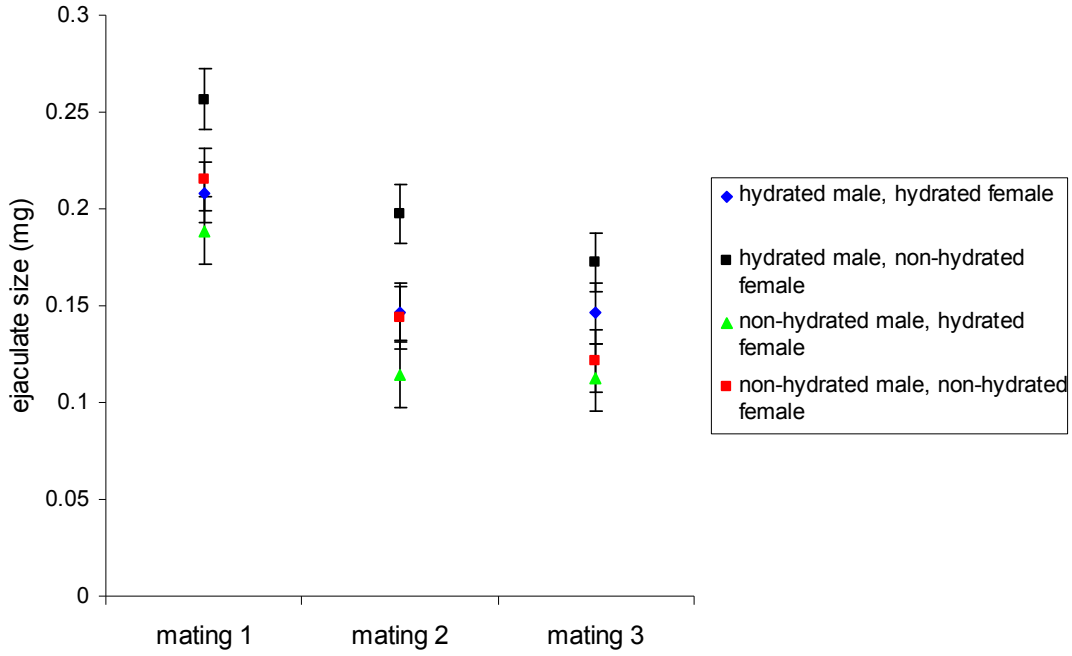

Figure 6.1: male hydration, female hydration and ejaculate size over three consecutive matings. Ejaculate size is given in milligrams, and is the mean value of all males within each cross-factored male and female hydration treatment, and is shown for each of three matings. Black squares represent hydrated males mated with non-hydrated females, red squares represent non-hydrated males mated with non-hydrated females, blue diamonds represent hydrated males mated with hydrated females, and green triangles represent non-hydrated males mated with hydrated females. Error bars show the standard error of the mean. For mating 1, hydrated male x hydrated female n (sample size) = 10, hydrated male x non-hydrated female n = 10, non-hydrated male x hydrated female n = 8 and non-hydrated male x non-hydrated female n = 9; for mating 2, hydrated male x hydrated female n = 10, hydrated male x non-hydrated female n = 10, non-hydrated male x hydrated female n = 8 and non-hydrated male x non-hydrated female n = 9; and for mating 3, hydrated male x hydrated female n = 10, hydrated male x non-hydrated female n = 10, non-hydrated male x hydrated female n = 8 and non-hydrated male x non-hydrated female n = 9.

Ejaculate sizes decreased with each subsequent matings in all males, irrespective of their hydration treatment ($F_{2, 68} = 0.20$, $p = 0.823$), or the hydration treatment of their female mates ($F_{2, 70} = 0.65$, $p = 0.526$).

6.4. Discussion

Male *C. maculatus* provided with water as adults produced larger ejaculates than males that were denied water. Regardless of their own hydration treatment, males allocated smaller ejaculates to females that were given water, than to females that were denied water. In all cases, ejaculate size decreased with each of three sequential matings; the rate of decrease in ejaculate size did not depend on whether or not males were given water, or whether they were mating with hydrated or non-hydrated females. Although I did not examine ejaculate content, because the only difference in experience between males in the two hydration groups was water provision, it is likely water is responsible for the increased size of ejaculates produced by hydrated males, although if males can use water to produce sperm or other ejaculatory resources, these might vary in quantity too.

The effect of water provision on male fitness has not been investigated in *C. maculatus*. Although I found increased ejaculate size as a result of giving males water, I cannot assume this would lead to increased male reproductive success. In Chapter 3, I found that larger ejaculates (as a result of adult male social context) did not lead to increased male reproductive success, whereas in Chapter 4, I found larger ejaculates (as a result of favourable larval conditions) did lead to increased reproductive success. The positive result in Chapter 4 was likely (at least partially) due to increased sperm numbers in larger ejaculates. Because, in the current study, hydration level is the only difference between males in the two treatment groups, it might be predicted that larger ejaculates in this case (presumably due only to increased water content) would not increase male reproductive success. However, since

increased ejaculate volume has been shown to increase female fecundity (Fox 1993; Savalli and Fox 1999; Edvardsson and Canal 2006), and delay female re-mating (Eady 1995), males in the current study might still gain paternity indirectly via ejaculate volume rather than sperm number. Further investigations into the effects of water provision on male reproductive success are required.

My finding that hydrated females receive smaller ejaculates than non-hydrated females could be explained by one of three hypotheses:

1. Because ejaculate size affects female fecundity and re-mating behaviour (Fox 1993; Eady 1995; Savalli and Fox 1999; Edvardsson and Canal 2006), males inseminate large ejaculates to increase their own reproductive success, and allocate larger ejaculates to non-hydrated females in order to achieve the same result (to compensate for the female's own lack of hydration). For this to be the case, males would be required to be able to assess female condition or hydration level. Female abdomen size decreases as the number of eggs females lay increases (personal observation); it is possible that these physical changes also occur as females become more dehydrated. It might therefore be possible for males to assess female hydration level when they come into contact before copulation. Because males have limited ejaculatory resources (Wedell *et al* 2002), they might allocate more water to a less-hydrated female, in attempt to provide sufficient resources to enable her to lay all her eggs, and to live long enough to achieve maximum fecundity. If a male perceives a female mate is well hydrated, he might inseminate only the minimum quantity of water required to carry his sperm, in order to save water resources for future matings. This hypothesis assumes copulation and ejaculate transfer are under male control.

2. Because of the beneficial effect of receiving larger ejaculates on female fecundity (Fox 1993; Eady 1995; Savalli and Fox 1999; Edvardsson and Canal 2006), non-hydrated females might allow insemination of larger ejaculates, in order to maximise their own fitness, as they

need more resources. One way in which females could receive larger ejaculates would be to copulate for longer (Edvardsson and Canal 2006). Although copulation has costs for female *C. maculatus* (Crudgington and Siva-Jothy 2000; Edvardsson and Tregenza 2005), for non-hydrated females, the benefits of receiving larger ejaculates might outweigh the costs of mating. Females have been shown to exhibit control over copulation in some insects (Linley 1975). During copulation in *C. maculatus*, females are damaged by barbs on male intromittent organs (Crudgington and Siva-Jothy 2000); before mating begins, females generally run away to avoid it (personal observation) and, once copulation is occurring, females kick with their back legs in attempt to dislodge males (Crudgington and Siva-Jothy 2000; Edvardsson and Tregenza 2005), particularly after copulation has been going on for some minutes (personal observation). It has been suggested that one of the functions of *C. maculatus* penis morphology is to anchor males within the female reproductive tract, so they can inseminate their ejaculate without being immediately kicked off (Crudgington and Siva-Jothy 2000). This might indicate that copulation, once it has commenced, is under male control, which is supported by the fact that copulation can continue for long periods of time despite females repeatedly kicking (personal observation). One possibility, however, is that this kicking behaviour is actually cryptic female choice (Eberhard 1996); females might attempt to avoid copulation by running, and try to shorten copulation by kicking, in order to only be inseminated by males that are fit enough to overcome these difficulties, therefore ensuring their offspring are fathered by the fittest males of the population. If this is the case, copulation might be under female control. There is some evidence that female *C. maculatus* exert some control over copulation duration (Crudgington and Siva-Jothy 2000); females prevented from kicking males off copulate for longer than those able to kick. Females in my study might therefore alter kicking behaviour to control copulation duration, and the volume of ejaculate they receive, in order to tailor the volume of ejaculate inseminated to meet their own need for water or other resources.

3. Males might always endeavour to mate for as long as possible, to inseminate as large an ejaculate as possible to maximise their fitness, and females are likely to try to mate for as little time as possible, to avoid the damaging effects of copulation (Crudgington and Siva-Jothy 2000); in this case, my result could be explained by the relative body conditions of males and females in the different scenarios. Non-hydrated males have less control over copulation than hydrated males, due to poorer condition, so always inseminate smaller ejaculates, perhaps due to shorter copulations (Edvardsson and Canal 2006). And non-hydrated females are less able to control copulation duration or ejaculate receipt by kicking than hydrated females, due to poorer body condition. So hydrated males always inseminate larger ejaculates than non-hydrated males, and hydrated females always receive smaller ejaculates than non-hydrated females. This hypothesis takes into account that copulation might be under both male and female control.

Ultimately, my experimental design does not allow me to tell these three hypotheses apart, so I cannot conclude whether my results are due to control of ejaculate size by males, or by females, or a combination of both. By measuring copulation duration in a future study, it might become apparent whether ejaculate size differences are due to differences in copulation duration, or whether ejaculates can be inseminated at different rates. It would be interesting to examine whether copulation duration differed between males mating with hydrated females and males mating with non-hydrated females. There is evidence in *C. maculatus* that ejaculate size is affected by copulation duration; ejaculate size increases with copulation duration (Edvardsson and Canal 2006). It is also possible that, by manipulating male and female hydration again, but also manipulating the ability of females to resist or control copulations by removing their kicking legs (Crudgington and Siva-Jothy 2000), it could become apparent whether hydrated females still receive smaller ejaculates than non-hydrated females. If they did, it would suggest ejaculate insemination was under male control, and that males might be tailoring the water content of their ejaculates to suit hydration levels of their female mates. This could be a fruitful course for future research.

In Chapter 3, I demonstrated that males in this population of *C. maculatus* do have the ability to react to perceived sperm competition levels, and plastically allocate ejaculate accordingly; males increased ejaculate sizes when sperm competition level was perceived to be high. In the current study, the only potential indication of sperm competition risk is female condition, since all females used were virgins, and all males were housed solitarily prior to mating. If female condition can be perceived, it is likely hydrated females would be judged to be in better condition than non-hydrated females, since hydrated females live longer (Edvardsson 2007) and are more fecund (Edvardsson 2007) than non-hydrated females. It would therefore be expected that, on perception of female hydration level, males would actually allocate larger ejaculates to hydrated females, which might represent a greater risk of sperm competition, due to their more desirable status as fitter females to rival males, in attempt to more effectively engage in sperm competition. Because I found males did not allocate larger ejaculates to females in better condition, this suggests males are not reacting to potential sperm competition level. Even non-hydrated males inseminated larger ejaculates into non-hydrated females than to hydrated females. These males have only metabolic water, from resources gained during larval development, to draw on. It could be that males are differentially allocating this small quantity of metabolic water on perception of female hydration level, or they might be differentially allocating other components of the ejaculate, including sperm. However, again if sperm competition risk was being assessed, I would expect the opposite result; males would allocate larger ejaculates to hydrated females, due to their better condition. It would be interesting to repeat the study but also count sperm, to examine whether water is indeed the only substance to be differently allocated in ejaculates of different size, or whether they contain different numbers of sperm too.

It is not known whether hydration increases male fitness in this species, although males given water do live longer than males denied water (personal observation). It would be interesting to investigate this further, by measuring differences in male fertility due to hydration level.

My finding that ejaculate size diminished with each of three sequential matings supports other findings in *C. maculatus* (Eady 1995; Savalli and Fox 1999). The rate at which ejaculate size decreased did not depend on whether males had been provided with water or not, or whether they were mating with hydrated or non-hydrated females. This suggests hydrated males maintained excess water at each of three matings, which is unsurprising given that their water was replenished between each mating. Ejaculate size has been shown to increase female fecundity in *C. maculatus* (Edvardsson and Canal 2006), suggesting there might be a fitness benefit to males of increasing ejaculate allocation. Whether the effect is due to ejaculate volume, or the quantities of ejaculatory components, requires further investigation; Eady (1994; 1995) found that increased sperm numbers delayed female re-mating, and Edvardsson and Canal (2006) found that increased ejaculate size increased female fecundity. Whether larger ejaculates in my study would lead to effects on female fitness is not known, but it is likely they are larger only because of excess water content. The finding that females provided with external sources of water as adults achieved increased fecundity (Edvardsson and Canal 2006) does suggest that the males in my study that are inseminating larger ejaculates might benefit via this effect, if females utilise water received via an ejaculate in the same way as water received through drinking. An important extension to this study would therefore be to investigate effects of male hydration on the re-mating behaviour and fecundity of their female mates.

Determining the ultimate effects of water provision on female *C. maculatus* has proved difficult, with different studies finding inconsistent results (Edvardsson 2007; Fox and Moya-Larano 2009). In some cases giving females water increased their fecundity and longevity (Edvardsson 2007) whilst in others it did not (Fox and Moya-Larano 2009). In all studies, however, providing females with water decreased their receptivity to re-mating (Edvardsson 2007; Fox and Moya-Larano 2009). Although I did not measure female fecundity or propensity to re-mate, personal observations suggest female longevity is increased with water provision. Further investigations into the effects of hydration on female fitness are required,

in order to establish whether water does benefit females, or whether only calorific nutrients increase female fitness, as has been suggested (Fox and Moya-Larano 2009).

In summary, water provision to both males and females affects ejaculate size; males given water produce larger ejaculates, and females given water receive smaller ejaculates. Neither of these results are unexpected, as individuals of this species develop on dried beans as larvae, then usually live without access to water as adults, and therefore are likely to be poorly hydrated. Males provided with external sources of water should therefore be less limited in the quantity of resources they can allocate to reproduction. Similarly, females provided with water are likely to be in better condition, so have lower requirement for resources via ejaculates. If ejaculate allocation is under male control males must have methods of perceiving female hydration state, and the mechanism by which they do so now warrants further investigation.

Chapter 7. Discussion

When sperm or other ejaculatory components represent a limited resource to a male, selection is expected to favour males that strategically allocate ejaculates in ways which maximise their fitness. However, unequivocal evidence for adaptive allocation in insects is limited, and a recent review highlighted the need for further studies into the fitness effects of plastic ejaculate allocation (Bretman *et al* 2011). In this thesis, I have explored how ejaculate allocation in male *Callosobruchus maculatus* is affected by both adult and larval conditions, and I also measured fitness effects of these ejaculate allocations from the males' perspective. The main conclusion of this work is that males of this species do not in fact show adaptive ejaculate allocation in response to cues about sperm competition level, as might be expected from aspects of their life-history and mating system. Although I demonstrated that males responded as predicted by sperm competition theory, by increasing ejaculate allocation in response to adult rival male presence, this increase in allocation did not lead to the expected fitness benefits for males, therefore suggesting the behaviour is not adaptive. When manipulating larval conditions, I found males did not increase their ejaculate allocation when larval density was high, suggesting that they were not responding to cues about sperm competition. Instead, males suffered a cost of larval crowding and produced smaller ejaculates, and fewer sperm, than males reared at low larval densities.

In this chapter, I link my findings with those in other insects, and assess whether there is in fact sufficient evidence that males show strategic (and adaptive) ejaculate allocation in response to cues about sperm competition level, in light of my results. I also explore the effects of two ecological factors on male reproductive traits and behaviours, and discuss how these might affect male fitness in this species.

7.1. Evidence for strategic ejaculate allocation

Because male *C. maculatus* mate multiply but have a finite supply of sperm and ejaculatory resources, I aimed to investigate what factors might cause males to alter their ejaculate allocations. Results from Chapters 3 and 4 suggest both adult and larval conditions affect male ejaculate allocation, but in different ways and with different consequences. In many species in which post-copulatory sexual selection affects male fitness, the number of rival males competing for fertilisation of the same set of ova can represent the risk (Gage 1991; Gage and Barnard 1996; Pound and Gage 2004; Nicholls *et al* 2001) or intensity (Schaus and Sakaluk 2001; Pilastro *et al* 2002; Pizzari *et al* 2003) of sperm competition a male's ejaculate is likely to face. To examine whether the perception of other adult males being around prior to copulation affected male ejaculate allocation, in Chapter 3 I manipulated adult male social context and measured ejaculate size. Adult rival male presence does indeed affect male ejaculate allocation; males experiencing the presence of four rivals prior to copulation consistently inseminated larger ejaculates than males that remained solitary prior to copulation. This is comparable with findings in other insects (Gage 1991; Gage and Barnard 1996), and suggests my male *C. maculatus* are reacting to cues about sperm competition risk, indicated to them by the presence of rival males. However, despite males appearing to behave in an adaptive way, male reproductive success was not affected by the ejaculate size changes demonstrated; males inseminating larger ejaculates in response to rival presence did not father more offspring, nor did they father a greater proportion of a clutch than a control competitor male, suggesting the observed ejaculate allocation tactic did not in fact represent an adaptive behaviour. This lack of effect was robust - the same null result occurred when measured using two methods of paternity assignment (sterile male technique and genetic markers), and using two different analyses (General Linear Models in Minitab and Generalised Linear Models in R). This result was unexpected, as it would be assumed a larger ejaculate would correspond to greater reproductive investment by males, which would therefore lead to increased reproductive success. In general, larger ejaculates might be

expected to increase male fitness in the face of competition, because if they contain more sperm, they might more effectively engage in sperm competition (Parker *et al* 1997), or they might displace sperm inseminated previously by rivals (Rondeau and Sainte-Marie 2001), or substances in them might stimulate female oviposition (Simmons 2001), or delay female re-mating (Kaitala and Wiklund 1994), or chemicals in them might adversely affect rival ejaculates (Harshman and Prout 1994). In *C. maculatus*, it has been previously demonstrated that larger ejaculates delay female re-mating (Eady 1995), and sperm numbers can be important in determining male reproductive success (Eady 1995), which makes my findings surprising as they are not consistent with this.

At first sight, this result might appear out of line with studies from other insect species, where apparently adaptive ejaculate allocation has been reported (Gage 1991; Gage and Baker 1991; Cook and Wedell 1996; Pound and Gage 2004; Yamane and Miyatake 2005; 2008). However, my results highlight the danger in assuming that just because a behaviour appears to be adaptive, it will necessarily have beneficial effects on male fitness. Typically, studies that have reported adaptive ejaculate allocation in response to cues of sperm competition risk have measured changes in ejaculate characteristics, but stopped short of examining directly the effects of these changes on male fitness (Gage 1991; Gage and Baker 1991; Cook and Wedell 1996; Pound and Gage 2004; Yamane and Miyatake 2005; 2008).

Why the increase in ejaculate size observed in response to social context in my study did not translate into increased reproductive success, despite the well documented effects of ejaculate size and sperm number on male fitness observed in other studies in this species (Eady 1994; 1995; Savalli and Fox 1999), is still unclear. Because I did not count sperm or measure the quantities of other components in the ejaculates (because necessary techniques were not available at the time), it cannot be determined whether these larger ejaculates contained more sperm. Because male reproductive success was not elevated in males producing larger ejaculates, this might suggest they did not contain more sperm than smaller ejaculates, or that

if there were more sperm, these were not effective at increasing male fitness, or that ejaculates were larger because of increased allocation of other non-sperm ejaculatory components. Results from my other chapters support the suggestion that ejaculate size might not always be important; the direct effect of larval density on male reproductive success over and above its effects via ejaculate size, and the finding that ejaculate size and sperm number are not linearly related, show the importance of ejaculate composition in addition to just its size.

If males were reacting to the presence of rivals by up-regulating other seminal products, results suggest these still did not work to increased male reproductive success under experimental conditions. Non-sperm ejaculatory components have been found to increase male reproductive success in some other insects (Svärd and Wiklund 1989; Rice 1996; Prout and Clark 2000; McNamara *et al* 2009). The literature suggests seminal fluid products in insects might increase male reproductive success by influencing female oviposition or re-mating behaviour (Simmons 2001). However, I found no effect of male social context on female receptivity in *C. maculatus*; males inseminating larger ejaculates in response to rival presence did not induce a longer period of non-receptivity in their female mates than males that were solitary prior to mating, and inseminated smaller ejaculates. Again this is surprising, given the previously-demonstrated effect of ejaculate size on female re-mating in this species (Eady 1995), and given the larger sample size, and hence statistical power, of my experiments. This might again suggest smaller ejaculates produced by solitary males in my experiment did not contain less of any effective seminal fluid product than larger ejaculates produced by grouped males. A potentially fruitful extension to my study would be to carry out similar adult social context manipulations, but, as well as measuring ejaculate size, count sperm numbers and, if techniques are available, assay other non-sperm components, to try and get to the bottom of why larger ejaculates in this instance did not increase male fertilisation precedence.

The results in this thesis suggest that variation in ejaculate size caused by some factors does affect male reproductive success, whilst variation due to other factors does not. Changes in ejaculate size due to larval density, and male mating history, and natural variation in ejaculate size within treatments due to male body size variation, all seem to affect male reproductive success. Conversely, variation in ejaculate size caused by adult social context does not seem to affect male reproductive success; this suggests that what appears to be the same male behaviour (alteration of ejaculate allocation) in different circumstances might actually have different underlying causes.

In Chapters 4 and 5, effects of larval density on ejaculate allocation and sperm number were investigated. I found, in contrast to what is predicted by sperm competition theory, that males experiencing the presence of conspecifics during development did not increase their ejaculate allocation during matings as adults. Rather, males reared at high larval density produced smaller ejaculates and fewer sperm. This contrasts with findings in the closely-related adzuki bean beetle, *C. chinensis* (Yamane and Miyatake 2005; 2008), in which males reared at high larval density actually produced more sperm than those reared at low density, when they belonged to strains with polyandrous mating systems. These contrasting results in such similar species suggest a fruitful course for investigation would be to further examine differences in ejaculates between the two species. In *C. maculatus*, both ejaculate size and sperm number decreased as a result of high larval density rearing; how does ejaculate size vary in *C. chinensis* males reared at different densities? Might other ejaculatory substances be down-regulated to allow increased sperm numbers in ejaculates produced by high larval density male *C. chinensis*? And, importantly, how do these differences in sperm numbers affect male reproductive success in *C. chinensis*? In light of my findings, it might be expected that high larval density male *C. chinensis* might achieve greater reproductive success than low density males, however this would need to be directly measured to be reliably determined, as my results from Chapter 3 highlight.

My findings highlight an important question; is there any robust evidence for strategic ejaculate allocation in response to sperm competition in insects, with demonstrable effects on male fitness? In a study using *Drosophila melanogaster*, Bretman *et al* (2009) demonstrated that males increase ejaculate allocation in response to the presence of rivals, and consequently achieve greater reproductive success (Bretman *et al* 2009). However, results might be confounded by the fact that males in the high sperm competition risk treatment were left to compete for the female in the mating arena; pre-copulatory sexual selection was therefore an additional selective force, so the males achieving matings in these cases would most likely be the strongest males of the group. It is unsurprising such males would achieve greatest fitness. In addition, a more recent study using *Drosophila* (Lizé *et al* 2012) found that males exhibited what appeared to be adaptive ejaculate allocation patterns, in response to sperm competition risks, even if they belonged to species without polyandrous mating systems. This result questions the relevance of ejaculate allocation in *Drosophila* species, and the robustness of the Bretman *et al* (2009) study, since males belonging to monandrous strains would not be expected to have been selected to react to cues about post-copulatory sexual selection, as females do not mate multiply. In addition, this study found no effect of male social context or reproductive allocation on female re-mating (Lizé *et al* 2012), suggesting that, like my *C. maculatus* males, male *Drosophila* that adjust their ejaculate allocation in response to sperm competition cues do not seem to gain fitness benefits. Therefore, determining whether plastic ejaculate allocation is actually adaptive in *Drosophila* requires more proof.

Few other studies have directly measured fitness consequences of male ejaculate allocation tactics in response to sperm competition levels; many have investigated male ejaculatory responses to different sperm competition scenarios, but have not followed through to the resulting effects on male reproductive success (Svärd and Wiklund 1986; Gage 1991; Gage and Baker 1991; Cook and Gage 1995; Cook and Wedell 1996; Gage and Barnard 1996; Simmons and Kvarnemo 1997; Wedell and Cook 1999; Schaus and Sakaluk 2001; Martin and Hosken 2002; Simmons *et al* 2007). In light of my findings, such studies should show

caution in the assumptions they make about the likely fitness consequences of observed male mating behaviours. Only by directly testing effects on male reproductive success in the same study as measuring ejaculate allocation, can it be reliably determined whether or not behaviours are adaptive.

7.2. Alternative explanations for ejaculate allocation patterns in response to adult social context

If the ejaculate allocation behaviours I have found do not function adaptively to increase male reproductive success in response to cues about sperm competition risk indicated by rival male presence during adulthood (Chapter 3), there might be a number of other alternative explanations for the results. There is widespread evidence among many species that males do adjust their ejaculates in response to their socio-sexual surroundings (Svärd and Wiklund 1986; Gage 1991; Gage and Baker 1991; Cook and Gage 1995; Cook and Wedell 1996; Gage and Barnard 1996; Simmons and Kvarnemo 1997; Wedell and Cook 1999; Schaus and Sakaluk 2001; Martin and Hosken 2002; Simmons *et al* 2007). Why might this adjustment occur if it does not increase male fertilisation success?

7.2.1. Effects on female fitness aspects that were not measured

One possible explanation for my ejaculate allocation results in *C. maculatus* is that ejaculate size might affect some other aspect of male or female fitness that I did not measure - if females lay larger eggs, for example, in response to receiving larger ejaculates, this might lead to increased offspring viability or fitness, therefore increasing male reproductive success indirectly. Maternal effects have been shown to affect male reproductive characteristics and emergence size in *C. maculatus* (Savalli and Fox 1998; Gay *et al* 2009). Offspring from smaller eggs have been found to emerge at smaller body sizes (Fox and Savalli 1998). Males inseminating larger ejaculates might therefore benefit by fathering larger offspring, via an

effect on female egg size, although there is then a question of why males would do this in response to cues about sperm competition. Measuring egg sizes might give clues about whether this could be the case.

7.2.2. Trade-off of current against future reproduction

One reason males might adjust ejaculate allocation differently under different circumstances, which might still explain the patterns in ejaculate size I found, is if they are not actually reacting to sperm competition level at all, but instead are trading off current reproduction against future reproduction. If socio-sexual surroundings give males clues about the likely future number of matings they might achieve, they might adjust the quantity of resources they invest in a current mating accordingly. In terms of my results from Chapter 3, this might explain why males exposed to rivals before mating produced larger ejaculates than those remaining solitary. The presence of rival males, but no females, might be taken by males as an indication of male-biased population sex ratio; under these circumstances they might perceive a low likelihood of securing future copulations if females are rare. Consequently, they might invest all or most of the ejaculatory resources they have in the current copulation. Conversely, males remaining solitary prior to mating might have no prior perception of sex ratio and, as the first individual they come across is female, they might perceive a greater likelihood of securing future additional copulations, if females are more common. Consequently, they might allocate a smaller ejaculate and save more of their resources for future matings. This hypothesis could therefore explain the patterns of ejaculate allocation I found in Chapter 3. If larger ejaculates benefit female fitness, which has been suggested by previous studies (Fox 1993), then males could still benefit from inseminating larger ejaculates, without this actually being an adaptation to post-copulatory sexual selection. However, the fact remains that I found no effects on male or female fitness. Although this life-history trade-off hypothesis might not predict males to benefit in the face of sperm competition, males should still gain fitness benefits when they invest more in current

reproduction, by increasing fecundity of the females they mate with. Because I found no effect on female fecundity, and because I measured both relative and absolute male reproductive success, and found no effect of male social context, this hypothesis is unlikely to explain my results. Perhaps if, by inseminating larger ejaculates, males allow females to produce better quality eggs, which lead to better quality offspring, there might still be an undetected fitness benefit for males. It might be that males perceiving a low likelihood of encountering subsequent females invest more in current reproduction by inseminating a larger ejaculate, in attempt to benefit females so they are able to live longer and lay better quality eggs, whereas solitary males save some resources for potential future matings; a further experiment testing effects on female longevity and egg size, and offspring fitness, might help confirm this. There is some evidence in other insects that ejaculate allocation affects female fecundity and longevity (Gillott 2003). It has been previously shown in *C. maculatus* that males do indeed make life-history trade-offs; males investing more in early copulations suffer reduced longevity (Brown *et al* 2009). This suggests males of this species can trade off aspects of their reproductive effort, supporting the theory that they might also trade off current versus future reproduction, but, having found no detectable fitness benefit of larger ejaculates produced in response to rival male presence, my results cannot support this hypothesis.

It is possible, though, that patterns of ejaculate allocation seen in other insects, assumed to be adaptive responses to sperm competition level, might actually instead represent male trade-offs of current against future reproduction. Where males are found to increase ejaculate allocation in response to socio-sexual surroundings, and this behaviour is assumed to be a reaction to sperm competition levels (Svärd and Wiklund 1986; Gage 1991; Gage and Barnard 1996), it might be instead that males are trading off current against future reproduction, and so increases in ejaculate allocation might not always lead to the expected immediate fitness gains for males. Studies directly measuring male reproductive success

caused by male ejaculate allocations are therefore needed before it can be reliably determined what exactly males are reacting to - sperm competition or current versus future reproduction.

7.2.3. Laboratory conditions

It is possible male ejaculate allocation patterns in my *C. maculatus* population are in fact adaptive behaviours, with fitness benefits that would be apparent under natural conditions, but that were not measurable under the experimental conditions I used. For example, if females re-mate to get additional water or nutrients (Edvardsson 2007; Fox and Moya-Larano 2009), then it is conceivable that larger ejaculates will have a bigger effect if females are initially in poor condition, and have little effect on females in good condition. It might be that my laboratory females are in too good a condition to show any fecundity advantage of receiving a larger ejaculate, whereas females in poorer condition might demonstrably benefit from receiving larger ejaculates. An interesting extension of my study might be to subject females to more stressful conditions, and further examine whether receipt of differently-sized ejaculates does affect their longevity, fecundity or re-mating rate. This would fit with the finding in *C. maculatus* that females in nutrient-deprived conditions benefited from multiple matings, in terms of fecundity, whereas females that were not nutrient-limited did not benefit (Fox 1993). This might potentially be the case in studies using other insects too, as long-term laboratory populations are often used. Studies in various insects using females of varying condition, and investigating effects of ejaculate allocation on male fitness, might yield interesting results about whether male allocation behaviours are in fact adaptive, but this effect is hidden by females that are too healthy to need to limit their reproduction. However, conditions in my experiments are likely to be similar to natural conditions, as *C. maculatus* are a stored product pest, and so living in a box of dried beans in a laboratory at around 30 °C should not be vastly different from living in a store of dried beans in the tropics.

Because I did not provide adult females or males with water or food resources as adults, it is likely my experimental design did represent ecologically relevant conditions; my females are not likely to be in better condition than females in the wild, therefore if there was an effect of ejaculate allocation on females in this species, I would expect to find it in my laboratory population.

7.2.4. A non-adaptive behaviour?

Another possible explanation for the results is that males are indeed perceiving sperm competition risk and allocating ejaculates accordingly, but that this behaviour is not adaptive. An evolutionary lag might mean the behavioural plasticity persists in this species, but no longer influences male reproductive success. This would fit with recent findings in *Drosophila* species, in which patterns of ejaculate allocation conforming to sperm competition theory were exhibited even in species without polyandry (Lizé *et al* 2012), and female re-mating was unaffected by differential ejaculate allocation (Lizé *et al* 2012), suggesting that, although the behaviour is exhibited, it is unlikely to be adaptive. Reasons why adult plasticity of ejaculate allocation in *C. maculatus* might have once been adaptive, but no longer is, would require more investigation. Perhaps in the wild, larger ejaculates might increase male reproductive success, but my laboratory population has lost this effect due to genetic drift. Population genetic differences have been shown to influence *C. maculatus* behaviour (Savalli *et al* 2000). By carrying out similar studies, measuring both effects on ejaculate size and male reproductive success, in other populations of *C. maculatus*, and indeed in other insects, this might become clearer.

7.3. Costs of competition in *Callosobruchus maculatus*

Because *C. maculatus* develop inside dried seeds and beans, the closed nature of their early life conditions could have important consequences for their fitness as adults. A single black-

eyed bean has previously been shown to yield up to 12 adult *C. maculatus* individuals (Giga and Smith 1991). Because *C. maculatus* do not normally feed or drink as adults, resources acquired during larval growth are likely to be crucial in determining adult fitness; my results from Chapters 4 and 5 suggest this is indeed the case. Males reared with competition from conspecifics for resources during larval development produced smaller ejaculates, and achieved lower reproductive success, than males reared without larval resource competition. Larger ejaculates produced by low larval density males contained more sperm than smaller ejaculates produced by high larval density males. Taken together these results suggest the greater reproductive success achieved by low larval density males are likely to be due to the greater numbers of sperm they inseminate, and that it is these extra sperm that are at least partially responsible for ejaculates being larger. Results in Chapters 4 and 5 make sense in terms of *C. maculatus* ecology; having to share a bean with other larvae would be expected to limit the quantity of resources each larva could acquire, which would limit the number of sperm they could produce, due to energetic constraints, and the need to trade off reproductive investment against somatic growth. However, my results were slightly surprising in the light of findings in the closely related species, *C. chinensis*, in which males of polyandrous strains reared at high larval density actually produce more sperm than those reared at low larval density (Yamane and Miyatake 2005; 2008). It seems *C. maculatus* are constrained by resource limitations when reared at high density, whereas *C. chinensis* males perceive high larval density as an indication of elevated sperm competition risk, and increase sperm numbers in accordance with theory. Due to their genetic and ecological similarity, this raises questions about why such differences have been found in the two species. One other notable difference in findings was the effect of larval density on male body size - in *C. chinensis*, males reared at high density emerged smaller (Yamane and Miyatake 2005; 2008), yet in my *C. maculatus*, male body size was unaffected by larval density. It might be that body size is more important than sperm number in *C. maculatus*, but the opposite is true in *C. chinensis*. If securing a mating is more difficult in *C. maculatus*, males with larger bodies might achieve greater reproductive success regardless of investment in sperm. However, this would be

unexpected, because of the relative degree of sexual size dimorphism in the two species - in *C. maculatus*, males are much smaller than females (Southgate *et al* 1957), whereas in *C. chinensis*, the sexes are more equally sized (Southgate 1958). This might suggest males have been selected to reach a certain size in *C. chinensis* in order to successfully copulate with females, whereas in *C. maculatus*, males have not been selected to grow as large as females. It is therefore surprising that my results suggest *C. maculatus* males maintain body size at the expense of investment in sperm, whereas in *C. chinensis* males appear to invest in sperm at the expense of body size. It might be that, in *C. maculatus*, there is a threshold body size below which copulation is impossible, and therefore minimum body size is maintained at the expense of investment in reproduction, in males reared with larval resource competition. Yamane and Miyatake (2005; 2008) also demonstrated that males of monandrous strains of *C. chinensis* reared at high density produce fewer sperm than those reared at low density; the same results as I found in *C. maculatus*. My strain of *C. maculatus* is polyandrous, so selection would be expected to work in the same way as in polyandrous strains of *C. chinensis*. This dramatic difference in sperm allocation pattern between two such closely related species certainly requires more examination.

Another aspect of their ecology that could have potentially important effects on male and female fitness is water availability. In the wild, because *C. maculatus* typically inhabit dried grain stores in arid habitats, they often live with limited or no water availability as adults, obtaining all their resources during larval development in dried beans. It has been previously demonstrated in *C. maculatus* that females provided with water as adults live longer (Edvardsson 2007), have greater fecundity (Edvardsson 2007), and re-mate less readily (Edvardsson 2007; Fox and Moya-Larano 2009) than females denied water. I was interested in this from the male point of view, so, in Chapter 6, I investigated how water provision for both males and females affected the sizes of ejaculates males inseminated during matings. Males given water produced larger ejaculates than those denied water, and females given water received smaller ejaculates than those denied water. Water provision allowed males to

increase their investment in reproduction; because ejaculate size has previously been shown to affect male reproductive success in this species (Eady 1994; 1995; Savalli and Fox 1999), it might be expected that hydrated males would achieve greater fitness than non-hydrated males. However, this cannot be assumed, particularly in light of the lack of such an effect of ejaculate size in Chapter 3. In addition, because males experienced the same larval conditions irrespective of their water treatment, it is likely their ejaculates differed only in water content and not, for example, sperm number, or quantity of other seminal resources (although this might be possible if males can use the extra water to make more sperm or seminal products), so direct effects of ejaculate size on male egg fertilising ability might not occur. However, additional water could bulk out ejaculates sufficiently to achieve the delay in female re-mating previously demonstrated in this species (Eady 1995). An interesting extension to my study would be to carry these hydration manipulations through to investigation of effects on male reproductive success; time constraints meant this was not possible at the time.

7.4. Who controls ejaculate allocation in *Callosobruchus maculatus*?

In Chapter 6, the size of the ejaculates females received depended on their own hydration levels, irrespective of the hydration levels of their male mates. This result could be explained by a number of hypotheses: if copulation is under male control, males might allocate larger ejaculates to non-hydrated females, in attempt to influence female re-mating and fecundity; if copulation is under female control, non-hydrated females might allow insemination of larger ejaculates, in order to benefit from extra resource provisioning; or if copulation is not under sole control of either sex, hydration level (and resultant body condition) might determine which sex has more control over mating (hydrated females try to terminate mating and hydrated males try to prolong it). Ultimately, the results of my study cannot determine which of these explanations is behind the effect of female hydration on ejaculate size. If copulation is under male control, the differential allocation of ejaculates to females of different hydration levels necessitates that males can detect female condition or hydration level. Female

abdomen size decreases as the number of eggs females lay increases (personal observation); these physical changes might also occur as females become more dehydrated, which might enable males to assess female hydration level when they come into contact before copulation.

There is evidence in *C. maculatus* that copulation might, at least partially, be under female control (Crudgington and Siva-Jothy 2000; Edvardsson and Tregenza 2005). Females prevented from kicking males during copulation (by having their rear legs removed) copulated for longer than intact females (Crudgington and Siva-Jothy 2000), suggesting females can control copulation duration by kicking. In relation to my study, this might suggest hydrated females are able to kick more effectively, due to being in better body condition, therefore possibly terminating copulation earlier, and hence receiving smaller ejaculates.

It would be difficult to design an experiment that could unequivocally determine whether females or males ultimately control copulation duration and ejaculate transfer, but the evidence that females are at least partially in control of copulation duration (Crudgington and Siva-Jothy 2000) again questions whether male ejaculate allocation patterns always represent adaptive behaviours exhibited by males. In this case, although results are consistent with males allocating ejaculate differently depending on female hydration, this cannot be determined; females might be in control. Carrying out a study in which copulation duration is measured, along with ejaculate size, in response to water provision, might shed light on whether copulation duration entirely explains ejaculate size differences. There is evidence in *C. maculatus* that ejaculate size increases with copulation duration (Edvardsson and Canal 2006). Perhaps by manipulating female hydration and female kicking ability it could be determined whether hydrated females, that are unable to kick, still receive smaller ejaculates than non-hydrated females; if so, this would suggest males exert at least some control over copulation, and could be allocating ejaculates strategically in response to female condition.

7.5. Potential extensions to this work

While this thesis begins to understand the relative importance of different aspects of male life experience on ejaculate allocation and consequent reproductive success, there is much still to establish. Reasons behind the demonstrable effect of adult social context on ejaculate allocation, but the lack of resultant effects on male reproductive success, will need more investigation. Possibly, by carefully examining ejaculates of different sizes, and potentially assaying their components, it could be established why larger ejaculates produced by males without larval resource competition, that contain more sperm, lead to greater male reproductive success, whereas larger ejaculates produced by males in response to adult social context do not lead to greater reproductive success. Another interesting addition to the study would be to examine the effects of different ejaculate allocations on male mortality, by measuring the effects of producing ejaculates of different size on male longevity. It seems unlikely that an advantage to males of producing larger ejaculates in response to adult social context, that I did not pick up, could be increased male longevity (in fact, the opposite would be expected), but investigations into male mortality could yield information on just how energetically demanding it is for males of this species to increase the size of their ejaculates. If it is found males responding to adult post-copulatory sexual selection cues by increasing their ejaculate allocation die sooner, this would suggest there is some component of male fitness that is improved by increasing ejaculate size, but that I have missed in this study - otherwise, why would they do it?

Similarly, effects of the receipt of ejaculates of different sizes on female mortality would be interesting to investigate, and could potentially explain why males increase ejaculate allocation even if they do not seem to benefit themselves - if females receiving larger ejaculates live longer, they might achieve greater lifetime fecundity, and so males might benefit by proxy. However, that I found no effect of male ejaculate allocation on total female fecundity, suggests this is unlikely.

Although *C. maculatus* is a suitable study organism for investigations into post-copulatory sexual selection, and is easy to work with, I hope the sometimes unexpected findings of this thesis will stimulate further similar studies in other insects, and even perhaps birds, mammals and fishes. Sperm competition theory attempts to explain reproductive resource allocation in all creatures, but it could be small differences in biology that explain the contrasting findings of my work and that of others (Yamane and Miyatake 2005; 2008). It is therefore important to properly examine male ejaculate allocation behaviour, even if patterns found correspond with theoretical predictions, to firmly establish whether they result in the expected consequences for male reproductive success. Without specific proof, and without linking the stages of demonstration of behavioural plasticity with resulting male fitness in the same study, the findings of my thesis suggest ejaculate allocation cannot always be assumed to be adaptive.

7.6. Conclusions

My thesis shows that various aspects of male *C. maculatus* life experience can influence their ejaculate allocations to matings - fixed effects of larval resource competition and water provision on ejaculate size, and plastic responses of ejaculate allocation to matings depending on adult rival presence. Only some of these factors, however, lead to changes in ejaculate that actually affect male reproductive success. This questions whether male reproductive behaviours, that are exhibited widely among many different species, are always adaptive. These findings add to the ever-growing field of post-copulatory sexual selection in insects, suggesting reasons behind certain reproductive behaviours, and potentially giving clues about what might happen in similar insects with comparable mating systems and life-histories. Because sexual selection is such a strong and important force, visibly shaping many aspects of most species, the post-copulatory sexual selection literature has focussed on which aspects of reproductive effort are shaped by it, and which factors lead to alterations in behaviour. The

findings of my thesis suggest that an additional, and equally important, factor to investigate is the end result of sexual selection - which males actually achieve greatest reproductive success.

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Appendix

This is a piece I wrote for the Wellcome Trust / Guardian Science Writing Prize 2011, which is about sperm competition in insects. It was short-listed in the professional scientists category.

The entry was published online on the Wellcome Trust blog:

http://wellcometrust.wordpress.com/2012/01/19/monogamy-is-easy/

Monogamy is easy

It's hard enough having to spread yourself thinly during your normal daily activities – work, sustenance, childcare, rest; the list goes on. Luckily for us (normally) monogamous types, our efforts in the bedroom are most often directed towards one individual. Imagine, though, the dilemma of having to divide your reproductive resources between partners. If you were a male seed beetle, *Callosobruchus maculatus*, you might face this very problem. You would have a limited supply of ejaculate, numerous females of differing ages and reproductive states, lots of rival males, and about a week to live. To fulfil your evolutionary potential and achieve reproductive success you need to prioritise your sexual encounters – do you allocate a little of your seed to each of several different females offering fairly decent returns, or do you use up all your sperm on one ripe, virgin female in the hope of fertilising each one of her hundreds of eggs?

Sperm is not a limitless resource. Males often have to use it economically to maximise their lifetime reproductive success. In many insects the situation is complex because females store sperm internally from several different mates, much of it surplus to requirement, so not all

males that achieve copulation can be guaranteed paternity. Males can sometimes bolster their chances, though, by adopting certain strategies to overcome this sperm competition.

As a promiscuous insect it is essential to assess your surroundings. If you were, say, a male cricket, *Gryllus veletis*, you might want to allocate lots of sperm when copulating if there is another male awaiting his turn with the female, in attempt to father a greater share of the resultant clutch than he does. If there are ten rival males around, though, you'd probably be better holding onto your ejaculate for now and saving your sperm for other, less competitive situations.

Now you're a bushcricket, *Kawanaphila nartee*. That nice, large female you can see might look appealing and you might think she has a lot to offer in terms of egg number and offspring quality. However, all the males think that. If you all mate with her a lot of you will lose out because she can't use all your sperm. It might be wiser to reduce your sperm allocation and instead offer more of it to a smaller, less desirable female. With her, your sperm will be unlikely to face competition and you'll probably father all her offspring.

Age, too, matters when it comes to females. If you were a meal moth, *Plodia interpunctella*, you'd assess female age upon mating and allocate your resources accordingly. Give more sperm to young females – they have more eggs in storage and more time to lay them. Your resources might be wasted on old females who could die before getting the chance to use your sperm on their few remaining eggs.

You also need to think about the non-sperm constituents of your ejaculate – water, nutrients and other chemicals. As a locust, *Locusta migratoria*, you could flush out sperm inseminated by males that have gone before you by allocating a large volume of water to your semen. It you were a swallowtail butterfly, *Papilio machaon* you could delay a female copulating

subsequently with a rival by inseminating a large ejaculate; she'll be too full to accept another mating for a while.

Don't forget about seasonality – the reproductive worth of females can change with the weather. As a small white butterfly, *Pieris rapae*, you can judge how many sperm and what quantity of nutrients to invest in a female depending on the period of the mating season. If you're quick off the mark females you encounter are likely to be virgins so you can inseminate just enough sperm to fertilise all their eggs but lots of nutrients to provide nourishment for your resultant offspring. Conversely, later in the season when females will have already mated with rivals, you should allocate more sperm but fewer nutritional resources – greater numbers of sperm will out-compete those of your rivals but there's no point spending nutrients on offspring that might not be yours.

With all these things to consider you might be glad not to be an insect. If you are indeed a monogamous type things might seem more black and white. You might feel more empathetic to the faithful Adélie penguin, *Pygoscelis adeliae*. Little does your partner know, however, that while allocating small numbers of sperm to her you are saving most of them for sneaky matings with others, in attempt to spread your genes far and wide.

As a male *Homo sapiens* you might think you have no control over the attributes of your ejaculate. However, with some suggestion that sperm numbers are increased when men return to a female partner having been away for a lengthy period, you might have more in common with an insect than you thought.